U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

universidade de aveiro
theoria poiesis praxis

MARINE SURFACE MICROLAYER AS A SOURCE OF ENTERIC VIRUSES

Ana Catarina Bispo Prata

Tese de Doutoramento em Ciências do Mar e do Ambiente

2014

Ana Catarina Bispo Prata

MARINE SURFACE MICROLAYER AS A SOURCE OF ENTERIC VIRUSES

Tese de Candidatura ao grau de Doutor em Ciências do Mar e do Ambiente Especialidade em Oceanografia e Ecossistemas Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto. Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas de Abel Salazar e Faculdade de Ciências) e da Universidade de Aveiro.

Orientador – Doutor Adelaide Almeida

Categoria – Professora Auxiliar

Afiliação – Departamento de Biologia da Universidade de Aveiro

Co-orientador – Doutor Newton Carlos Marcial Gomes

Categoria – Investigador Principal do CESAM

Afiliação – Departamento de Biologia da Universidade de Aveiro

LEGAL DETAILS

In compliance with what is stated in the legislation in vigor, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results here stated, as well as in their interpretation and writing of the respective manuscripts.
This thesis includes one scientific paper published in an international journal and three articles in preparation originated from part of the results obtained in the experimental work referenced as:

- Prata C, Ribeiro A, Cunha A , Gomes NCM, Almeida A, 2012, Ultracentrifugation as a direct method to concentrate viruses in environmental waters: virus-like particles enumeration as a new approach to determine the efficiency of recovery, Journal of Environmental Monitoring, 14 (1), 64-70.

- Prata C, Cunha A, Gomes N, Almeida A, Surface Microlayer as a source of health relevant enteric viruses in Ria de Aveiro.

- Prata C, Cunha A, Gomes N,Nunes, T, Almeida A, Marine aerosols as a source of health relevant virus.

- Prata C, Coelho F, Ladeiro B, Cunha A, Gomes N, Almeida A, Influence of UV light exposure on viral abundance.

Table of Contents

Agradecimentos

Esta viagem termina agora e surge o momento de agradecer aos que passaram pelo meu caminho, durante estes cinco anos, contribuindo de algum modo para a realização deste trabalho. A todos o meu mais sincero obrigada.

Começo por agradecer à minha orientadora, Dra Adelaide Almeida e ao co-orientador, Newton Gomes, pela disponibilidade mostrada ao longo de todo o trabalho, pela ajuda na resolução de problemas e pela opinião crítica relativamente a todas as questões de trabalho.

Aos colegas de laboratório, pela ajuda dada em campo, no laboratório, ou simplesmente por descontraírem o ambiente quando era necessário.

Aos amigos promarianos que desde o primeiro dia se mostraram disponíveis para ajudar em tudo o que fosse necessário. Juntos inicámos esta viagem e juntos estamos a terminá-la (uns mais cedo do que outros). Muito obrigada por estarem sempre presentes: Allan Souza, Fátima Pinto, Helena Oliveira, Inês Rosa, Joana Rocha, Martina Ilarri, Rita Polónia, Rosa Reboreda, Rute Pinto e Sérgio Prats.

Agradeço aos melhores pais do mundo, os meus, por acreditarem em mim, por me terem apoiado em mais esta jornada da minha vida e por estarem lá, sempre que preciso.

A todos os meus amigos, mas em especial à Sónia, ao Francisco, ao João e à Inês, por me terem apoiado, ouvido os desabafos e dado força nos momentos difíceis, para seguir em frente.

À Mariana, ao André e à família Antunes, por terem entrado na minha vida e marcado a diferença. Obrigada do fundo do coração.

Ao Henrique, por teres reaparecido na minha vida, por me fazeres acreditar que há algo melhor. Obrigada por seres assim, perfeito para mim.

Figures Index

Tables Index

Abreviations List

AGI	All glass impingers
BWZ	Brackish water zone
CPE	Cytophatyc effects
DNA	Deoxiribonucleic acid
ds	Double strand
ELISA	Enzime-linked immunocorbent assay
ELSS	Experimental life support system
HAV	Hepatitis A virus
ICC	Integrated cell culture
ICTV	International Committee of Taxonomy of virus
IFA	Immunofluorescence assay
MZ	Marine zone
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
PAR	Photosynthetically active radiation
PCR	Polimerase chain reaction
PEG	Polyethyleneglycol precipitation
qPCR	Quantitative Polimerase chain reaction
RNA	Ribonucleic acid
SML	Surface microlayer
ss	Single strand
TFF	Tangencial Flow Filtration
UVR	Ultra Violet Radiation
UW	Under water
UVA	Ultra Violet Radiation A
UVB	Ultra Violet Radiation B
UVC	Ultra Violet Radiation C
VFF	Vortex Flow Filtration

Resumo

Os vírus entéricos são um importante grupo de agentes patogéncicos, responsáveis por um grande número de doenças no Homem. Podem existir naturalmente no ambiente aquático ou ser transportados até lá. Alguns destes vírus são considerados emergentes no ambiente aquático, representando um elevado risco para a saúde pública, economia e ecologia. Assim, é de extrema importância, conhecer os seus reservatórios naturais, bem como o modo como podem ser transferidos a partir do ambiente aquático para a atmosfera, a fim de minimizar os riscos por eles provocados.

Apesar do elevado número de vírus presente no ambiente aquático, apenas uma pequena parte causa infeções no Homem, mesmo quando presentes em baixa concentração, uma vez que apresentam uma baixa dose infeciosa.

A concentração das amostras de água representa um importante passo aquando da deteção dos vírus entéricos. No entanto, não existe um método ideal para concentrar vírus em amostras de água, dependendo sempre do objetivo do trabalho em questão. Com este trabalho, verificou-se que o método da ultracentrifugação é um bom método para a concentração de águas ambientais uma vez que foram obtidas taxas de recuperação entre 66 e 76%. Por outro lado, este método não adiciona compostos químicos, o que permite usá-lo para detetar posteriormente vírus usando métodos moleculares.

A microcamada superficial (SML) constitui a interface entre a água e o ar, acumulando microrganismos e partículas. Sabe-se que constitui um reservatório natural para vários microrganismos, a partir do qual são libertados aerossóis para a atmosfera, levando assim os materiais acumulados na SML, incluindo vírus. Foram obtidos neste trabalho valores de enriquecimento de vírus entéricos na SML, relativamente à água subjacente (UW), superiores a 2, tanto na zona marinha como na zona salobra. Comparando esses valores para a SML, relativamente aos aerossóis, o enriquecimento é de cerca de 26 na zona marinha e cerca de 30 na zona salobra, mostrando assim que a SML funciona como reservatório para os vírus entéricos e que estes são transmitidos, do ambiente aquático para a atmosfera, através dos aerossóis.

Relativamente à abundância dos vírus entéricos estudados, observou-se que rotavirus e enterovirus são mais abundante nos meses mais frios e que HAV apresenta abundância semelhante nos meses quentes e frios. As variações na abundância dos vírus entéricos foram explicadas pelas variações de temperatura da água e pela concentração de nitratos e de nitritos, o que sugere que estes vírus têm origem

terrestre, sendo transportados para o ambiente marinho através das águas de escorrência provocadas pelas chuvas, durante os meses mais frios.

Atualmente as alterações climáticas e as suas consequências nos ecossistemas são consideradas um problema grave, pelo que foi testada neste trabalho a influência da exposição à luz UV, na abundância de vírus entéricos de RNA. Observámos que os rotavírus foram mais resistentes à UVR do que os enterovirus ($p = 0.03 < 0.05$ and $p = 0.41 < 0.05$, respectivamente), podendo as diferenças observadas entre os dois grupos estar relacionada não só com o tipo de RNA, dsRNA nos rotavirus e ssRNA nos enterovirus, mas também com a estrutura da cápside, constituída por três camadas nos rotavírus e apenas uma nos enterovírus.

Abstract

Enteric viruses are an important group of pathogenic agents responsible for a large number of diseases in humans. They can exist naturally in the aquatic environment or be transported from terrestrial environment to the water. Viruses present in this group are currently considered emerging in the aquatic environment, representing a hazard to public health, economy and ecology. It is therefore of outmost importance to know their natural reservoirs, as well as how they can be transferred from the aquatic environment to the atmosphere in order to minimize the risks posed by them.

Despite the high number of viruses present in the aquatic environment, only a small part is responsible for human infections. However, even at low concentrations viruses are responsible for infections, since they have a low infectious dose.

The concentration of water samples is an important step for the detection of enteric viruses. However, there is no ideal method to concentrate viruses in water samples, always depending of the goal of the work. In this work, we found that the ultracentrifugation method is a good method for the concentration of viruses in environmental waters, once it was obtained recovery rates between 66 and 76%. On the other hand, this method does not add any chemical compound, allowing posterior viral detection by molecular techniques.

Surface microlayer (SML) corresponds to the interface between water and air, accumulating microorganisms and particles. It is known that constitutes a natural reservoir for enteric viruses from which aerosol are released, transporting materials accumulated in the SML. In this study the enrichment values for SML in respect to underwater (UW) were higher than 2 in the marine zone and in the brackish water zone. The enrichment of SML regarding to aerosols, was about 26 in the marine zone and about 30 in the brackish water zone for the SML, showing that the SML acts as a reservoir for enteric virus and that these are transmitted from the aquatic environment into the atmosphere through aerosols.

Regarding the abundance of enteric viruses studied, rotavirus and enterovirus were more abundant during cold months and HAV presents similar abundance in cold and warm months. Variations in the abundance of enteric virus were explained by differences in water temperature and in nitrates and nitrites concentration, suggesting terrestrial origin of these viruses, wich are transported to maritime system by rain water runoff, during cold months.

Nowadays we face serious problems of climate change and the consequences of changes in the ecosystems are a major problem. The influence of UV light exposure in the abundance of enteric viruses was tested and it was found that only enterovirus showed a significantly reduction in their abundance ($p = 0:03 < 0.05$ and $p = 0:41 < 0.05$, respectively). Differences observed betwenn the two groups may be related with the RNA type, dsRNA for rotavirus and ssRNA for enterovirus, but also with the capside structure, with three layes for rotavirus and only one for enterovirus.

Thesis Outline

The viruses in the aquatic system are among the best fitted living beings to become emerging pathogens and are able to survive and remain infectious for long periods. Most of these microorganisms present low infectious dose, representing a major threat for human health and a problem for economy and environmental ecology.
Regarding to this problems, it becomes urgent to determine the source of viral contaminations and the way of their transference and transport, in order to evaluate their association with health risk and implement the required measures.

This work pretends to understand how surface microlayer (SML) acts as a reservoir for enteric viruses in aquatic system, the way they are transferred to the atmosphere and the influence of UV light on viral abundance. In order to achieve the knowledge pretended, several specific goals were established:

- To determine the best concentration method to detect viruses by molecular methods.
- To determine the role of SML as a reservoir of enteric virus in the aquatic system.
- To determine the best method for aerosols formation and recovery.
- To determine the role of aerosols in the transference of enteric viruses from the aquatic environment to the atmosphere.
- To determine the influence of UV light on enteric viral abundance in the aquatic system.

Chapter 1 presents a careful revision of the state of the art for enteric viruses in the aquatic system, the factors affecting their survival, the way they are transmitted to the atmosphere and the methods used to detect them.
Since only a small number of viruses are epidemiologically relevant to human health, direct viral detection is not possible, being necessary to concentrate large volumes of water before proceeding to their detection. There are several concentration methods for viruses but none of these methods is considered perfect. Since the choice of the concentration method depends on the sample tested and the subsequent analysis to be made.

Chapter 2 includes a laboratorial work part, to compare two concentration methods (organic flocculation and ultracentrifugation) and decide which one would be used in the subsequent work.

Chapter 3 includes a study on enteric viral abundance in both SML and underwater (UW) in order to determine the role of SML and aerosols in accumulation and transference of enteric virus.
In virology, much is already known about the viruses present in the aquatic system that represent a health hazard for humans. However, little is known about their natural reservoirs, namely the SML.
In this chapter it was also evaluated the physical and chemical parameters that may affect viral abundance.

Chapter 4 includes a laboratorial work section to develop a protocol for aerosols collection and a field work section, where aerosols were collected and viral abundance determined. It is known that aerosols constitute the main vector for transport of viruses across the air-water interface, being their composition dependent on SML composition.

Chapter 5 presents a laboratorial study about the UV light influence on enteric viruses abundance. Viral survival may vary, depending on viral type. However, there are several factors that may affect that survival.

Chapter 6 presents all the conclusions of this thesis and some future work considerations.

Chapter 1 - Introduction

1. Introduction

At the present, poor water quality due to the presence of viruses still represents a major threat for human health (Symonds and Breitbart, 2014). It becomes urgent to trace and characterize the type and origin of viral contamination in order to evaluate their association with health risk and implement the required measures (Bosch 1998, Griffin *et al*, 2003, Bosch *et al.* 2005, Myrmel *et al*. 2006).

Viruses are the simplest form of life, consisting of genetic material, which may be either DNA or RNA, surrounded by a protein coat and, in some cases, by a membranous envelope, being characterized by their simple organization and their unique mode of replication (White *et al*, 1994, Mahony *et al*. 2008). Unlike cellular organisms, viruses do not contain all the biochemical mechanisms for their own replication; they need to use the biochemical mechanisms of a host cell to synthesize and assemble their separate components.

Viruses vary in their stability. Some are very stable and survive well outside the host body while others do not survive well and, therefore, usually require close contact for transmission being readily destroyed in the environment (White *et al*. 1994, Mahy *et al*. 2009).

Among microorganisms, viruses are best fit to become emerging pathogens since they are able to adapt not only by mutation but also through recombination and reassortment and can thus become able to infect new hosts and to adapt to new environments (Desselberger 2000, Tabor 2007, LaRosa et al, 2012). Mutations contribute to changes in viral pathogenicity, since viruses have a simple structure allowing mutations to occur easily and viral explosive replication magnifies the mutation (Tabor, 2007). RNA viruses are particularly susceptible to these modifications since their host lack molecular “proofreading” mechanisms to correct these mutations and replication errors (Tabor 2007; Domingo, 2010).

Some health relevant enteric viral groups are nowadays considered to be emerging waterborne pathogens (LaRosa et al, 2012; Woods, 2013), increasing the concern about the discharge of human enteric viruses in not only fresh water but also in estuarine and marine waters (Lee and Kim, 2002; Hamza *et al*, 2009; Rodríguez-Díaz *et al*, 2009).

1.1. Enteric viruses in the aquatic system

Enteric viruses can be characterized as a group of viruses which may be present in the gastrointestinal tract causing disease or asymptomatic infection (Wyn-Jones and Sellwood 2001). Despite the existence of more than 140 types of viruses in wastewaters that can cause a variety of diseases to humans (hepatitis, gastroenteritis, meningitis, fever, influenza, respiratory disease, conjunctivitis, among others) (Goyal *et al*, 1984; Puig et al, 1994; Bosch 1998; Pianetti *et al*, 2000;Griffin 2003; Bosch *et al*, 2005;Pusch *et al*, 2005; Bocsh et al, 2008; Hamza et al,2009), only a small number of viruses is epidemiologically relevant (Bocsh, 1998). These viruses can exist naturally in the aquatic environment or could be transported to this environment through sewage outfall and vessel wastewater discharge (Grabow 1996, Pianetti *et al*. 2000, Griffin *et al.* 2003, Suttle 2005, Bosh *et al*. 2006, Suttle 2007, Lugoli *et al*. 2009). The pathogenic viruses are able to survive for long periods of time in the aquatic environment especially when they are bound to organic material of fecal origin (Grabow 1996, Griffin *et al.* 2003, Bosh *et al*. 2006, Suttle 2007, Lugoli *et al*. 2009). Although they can survive in water, as they are obligate intracellular parasites they cannot multiply in the environment (Wyn-Jones and Sellwood 2001). The majority of pathogenic viruses that represent a significant public health threat and are emerging in the marine environment are transmitted via the fecal-oral route and belong to the families *Picornaviridae* (Enterovirus and hepatitis A virus (HAV), *Caliciviridae* (norovirus and sapovirus); *Adenoviridae* (adenovirus strains 3, 7, 40 and 41) and *Reoviridae* (rotavirus) (Bocsh et al, 2008; Percival et al., 2004; Rodrıguez-Dıaz et al, 2009; LaRosa et al, 2012). Moreover, most viruses in the marine environment can remain infectious in sediments for long periods that goes from decades to hundreds of years (Griffin *et al*. 2003, Fong *et al*. 2005, Bosch *et al*. 2006, Bosch *et al*. 2008; LaRosa et al, 2012).

As most enteric viruses have a low infectious dose of 10-100 particles or possibly even less, they represent a potential problem to public health, economy and environmental ecology, in areas used for recreational purpose or from which shellfish are harvested for human consumption (Fong *et al*. 2005, Hamza et al, 2009; Lee and Kim, 2005; Rodrıguez-Dıaz et al, 2009). In fact, viruses have been isolated from seawater and shellfish that fulfill current criteria on bacterial indicators, revealing shortcomings in microbiological quality standards (Lugoli *et al*. 2009). Despite the fact that several reports describe a lack of correlation between bacterial indicator microorganisms and viruses, pathogenic viruses such HAV, norovirus, rotavirus and enterovirus may be detected in shellfish from areas classified as suitable for commercial exploitation

according to fecal coliform criteria (Le-Guyader 1983, Romalde 2002, Bosch *et al*. 2005; Mesquita et al, 2011).

***Adenoviridae* family**

The *Adenoviridae* family includes a group of icosahedral nonenveloped virus, ranging from 70 to 100 nm in diameter, with a double-stranded DNA genome of 30 to 40 kb, depending on the serotype (Bosch *et al*. 2006, Hundesa *et al*. 2006, Mahony *et al*. 2008, Acosta *et al*. 2009; Barlan et al, 2011).

These viruses are able to resist to extreme conditions, including unfavorable pH environments (they can survive to acidic pH of 5-6) (Pond 2005). Therefore, they can live outside of a host for long periods of time compared to other viruses (Pond 2005). Adenovirus has been shown to be resistant to both tertiary treatment and UV radiation (Pond 2005) and even with damaged DNA they can successfully infect host cells (Ko *et al*. 2003, Jothikumar 2005, Eischeid 2009).

There are five genera in this family, *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*, but only the genera Mastadenovirus could affect humans (Harrach et al, 2011, Joseph et al, 2014). In this genus there are 56 serotypes of human adenoviruses (HAdVs) categorized by hemagglutination into seven subgenera, A-G, based on their physicochemical, biological, immunological and genetic properties, that define their ability to agglutinate red blood cells. The HAdVs serotypes 40 and 41 (subgenera F) are critical etiological agents of viral gastroenteritis in children, frequently associated to waterborne transmition (Griffin et *al*. 2003, Ko *et al*. 2003, Fong *et al*. 2005, Eischeid *et al*. 2009; Barlan et al. 2011, Robinson et al, 2011; Lee et al, 2012).

Adenovirus have been shown to be prevalent in seawater and in shellfish (Jiang *et al*. 2001, Pina *et al*. 1998, Bofill-Mas *et al*. 2006; Corrêa et al, 2012; La Rosa et al. 2012; Ming et al, 2013). Some authors suggested that they may survive for prolonged periods in water, representing a potential route of transmission (Enriques et al, 1995; Lebecka et al. 2009; Rigotto et al, 2011). Their high incidence found in harvested oyster suggests that these viruses may be endemic in coastal marine environments and that people infected by adenovirus may act as carriers, shedding virus in their stools without showing any symptoms (Aragão *et al*. 2010).

This group of viruses is being seen as a high priority for possible future regulation (Griffin *et al*. 2003, Ko *et al*. 2003, Fong *et al*. 2005, Xagararaki *et al*. 2007) in Europe, and different authors have suggested its inclusion as an index of pollution of human origin (Linden *et al*. 2007, Muscillo *et al*. 2008; Vieira et al, 2012).

***Picornaviridae* family**

Viruses belonging to family *Picornaviridae* are nonenveloped with icosahedral capsid, ranging from 27 to 30 nm in diameter and a positive-sense single-stranded RNA genome of 7.2 to 8.4 Kb whose host range are typically restricted to mammals (Grohmann et al, 1997, Mahy *et al*, 2009, Voevodin *et al*, 2009).

The *Picornaviridae* family includes 17 genera (*Aphthovirus, Aquamavirus, Avihepatovirus, Cardiovirus, Cosavirus, Dicipivirus, Enterovirus (coxsackievirus, poliovirus and echovirus), Erbovirus, Hepatovirus* (hepatite A virus), *Kobuvirus, Megrivirus, Parechovirus, Salivirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus*), but only some of them, like *Enterovirus* and *Hepatovirus*, have been detected in aquatic environment (Griffin *et al.* 2003, Fong *et al*. 2005; Mahy *et al*. 2009; Chigor and Okoh, 2012; Moresco et al, 2012; Lim et al, 2014), being infections in humans reported to peak in summer and early fall, which also coincides with increased water recreational activities and water contact (Fong *et al*. 2005; Liu et al, 2014).

Medically important viruses of this family belong to Enterovirus, Hepatovirus, Rhinovirus, Parechovirus and more recently Kobuvirus (Voevodin *et al*. 2009) being the genera Hepatovirus one of the most implicated in outbreaks transmitted from the marine environment (Voevodin *et al*, 2009). Hepatite A virus (HAV) represents one of the most important public health problems in undeveloped countries (Croci *et al*, 2000; Bosch *et al,* 2005; Fong *et al*, 2005; Yeh *et al*, 2008), being the distribution patterns closely related to socioeconomic development (Divizia *et al*, 2004; Pintó *et al*, 2007). HAV are environmentally stable, and able to persist in cool, damp, and dark environments for periods of months, or even a year or more (Kingsley, 2013). They are resistant to low pH, detergents, and organic solvents, and are more resistant than bacteria to water treatments, such as chlorination (Kingsley, 2013). Hepatitis A is one of the most serious viral infections linked to shellfish consumption, causing a serious debilitating disease and even death and today they are considered emerging viruses in non-endemic areas (Halliday et al, 1991; Romalde et al, 2001; kingsley et al, 2002; Romalde et al, 2002; Guillois-Bécel et al, 2009; Ming et al, 2013; Bigoraj et al, 2014).

Although major enterovirus outbreaks of waterborne disease, are comparatively rare relatively to other viruses, there are substantial evidences that human enteroviruses are frequently present in recreational waters (Leveque *et al*. 2008).

***Caliciviridae* family**

Calicivirus have a nonenveloped, icosahedral capsid composed of a single protein type, ranging from 28 to 35 nm of diameter, with a single-stranded, non-segmented,

positive-sense RNA genome that varies in length from 7.4 to 7.7 kb, depending on the genotype (Grant 2010). They encode two or three Open Reading Frames (ORF), differently arranged, depending on the genera (Grant 2010). ORF1 encodes for the mature non-structural polyprotein, including the RNA-dependent RNA polymerase. ORF2 and ORF3 encode structural proteins, the major capsid protein (VP1) and a minor structural protein (VP2) respectively (White *et al*. 1994, Katayama *et al*. 2004, Asanaka *et al*. 2005, Grant 2010; Eden et al, 2013; Vega et al, 2014).

The *Caliciviridae* family includes five genera: *Vesivirus, Lagovirus, Norovirus, Sapovirus* and *Nebovirus* (Bosch 1998, Griffin *et al*. 2003, Fong *et al*. 2005, Bosch *et al*. 2008, Voevodin *et al*. 2009, Grant 2010; Mikalsen et al, 2014) with human pathogens belonging to the *Norovirus* and *Sapovirus* genera (Voevodin *et al*. 2009, Grant 2010; Clark et al. 2012).

Human caliciviruses (Genotypes II and IV) (Vega et al, 2014) are very resistant to inactivation, prolonged asymptomatic shedding, have great environmental stability (can remain more than 14 days at 15ºC seawater), and great strain diversity (Smith *et al*. 1993, Jothikumar *et al*. 2005, Constantini *et al*. 2006, Rosa *et al*. 2007, Le-Guyader *et al*. 2009). These characteristics allow them to circulate efficiently in both clinical and environmental contexts, increasing the risk of infection by members of this group (Jothikumar *et al*. 2005, Rosa *et al*. 2007, Le-Guyader *et al*. 2009). In fact, they have been detected in different water environments such as sewages, municipal water, well water, ice cubes, and recreational waters (Jothikumar *et al*. 2005, Rosa *et al*. 2007, Le-Guyader *et al*. 2009). The risk of infection after consumption of raw or improperly cooked seafood or after exposure to contaminated recreational water is so considered high for this group (Jothikumar *et al*. 2005, Rosa *et al*. 2007, Le-Guyader *et al*. 2009).

Norovirus is the leading cause of acute viral gastroenteritis and is estimated to cause almost half of all cases of gastroenteritis globally (Eden et al, 2013). A highly infectious pathogen, norovirus is readily transmitted from person to person or through contamination of water and food sources (Eden et al, 2013). It have been identified as the primary pathogens associated with shellfish-borne gastroenteritis in the developed countries in people of all ages (Smith *et al*. 1993, Nishida *et al*. 2003, Constantini *et al*. 2006, Rosa *et al*. 2007, Nenonen *et al*. 2008, Grant 2010).

Noroviruses are a group of genetically diverse viruses that can be classified into 6 genogroups (I–VI), but only genogroups I, II and IV have been found to infect humans (Hasing et al, 2014; Vega et al, 2014). Norovirus genogroup II genotype 4 (GII.4) is of high relevance to public health as about 60% of NoV outbreaks are caused by this genotype globally (Hasing et al, 2014). Norovirus GII.4 evolves rapidly resulting in new

genetic clusters or variants every 2–5 years that quickly replace previous circulating GII.4 strains (Eden et al, 2013; Leshem et al, 2013; Hasing et al, 2014; Vega et al, 2014). These new NoV strains have often, but not always, led to increased outbreak activity (Leshem et al, 2013). While some GII.4 variants such as Cairo 2007, Asia 2003 and Japan 2008 only circulated in limited geographic regions (Hasing et al, 2014), variants US95/96 1995, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009 and Sydney 2012 spread globally causing pandemic NoV outbreaks (Eden et al, 2013; Leshem et al, 2013; Hasing et al., 2014).

Like HAV, Norovirus are environmentally stable and able to persist in cool, damp, and dark environments for periods of months, or even a year or more. They are also resistant to low pH, detergents, and organic solvents, and are more resistant than bacteria to water treatments, such as chlorination (Kingsley, 2013).

***Reoviridae* family**

Reoviridae is one of the largest families of virus, characterized by a genome of 9–12 segments of linear, double-stranded (ds) RNA of 16 to 27 Kb of size (Attoui et al., 2005; Bos *et al,* 2004; Grassi *et al*, 2009). Viruses belonging to this family are non-lipid-coated icosahedral, ranging from 60 to 80 nm in diameter. Most of them have an outer capsid shell playing a primary role in cell attachment and penetration, and a structurally conserved core that contains the genome and facilitates endogenous transcription mechanism (Bos *et al,* 2004; Grassi *et al*, 2009; Lawton et al, 2000). The genome is enclosed by a triple-layered capsid composed of a double protein shell and an inner core. The innermost layer is composed by VP2 protein, the intermediate layer consists of VP6 protein and the outermost layer is composed of glycoprotein VP7 and the spike protein VP4 (Caballero *et al*. 2004, Bosch *et al*. 2006). The structural proteins VP7 and VP4 are important in virus infectivity (Bosch *et al*. 2006) and the VP6 protein is designated the group-specific antigen being the major target of rotavirus diagnostic assays (Bosch *et al*. 2006).

Since the 8th report of the International Committee on Taxonomy of Virus (ICTV), three new genera, *Mimoreovirus*, *Cardoreovirus* and *Dinovernavirus*, have been described for this family, with a total of 84 reoviruses within 15 genera have been defined under the family *Reoviridae* (Attoui et al., 2006; Harrach et al, 2011; Huang et al, 2012).

Rotaviruses are resistant to disinfectants, heat, proteolytic enzymes and pH values between 3 and 10 (Bosch *et al*. 2006, Grassi *et al*. 2009). However, they do not show the same tolerance to extreme conditions as other enteric viruses, although they are stable in the environment (Bosch *et al*. 2006, Grassi *et al*. 2009).

Some studies have reported the occurrence of rotaviruses in natural water, becoming the most important agent of gastroenteritis transmitted from the aquatic environment in this family (Bos *et al*. 2004, Loisy et al. 2004, Grassi *et al*. 2009). Environmental transmission frequently occurs through shellfish grown in polluted waters (Caballero *et al*. 2004; Grassi *et al*. 2009) and diseases caused by rotaviruses are more common during the winter months in countries with a temperate climate (Bosch et al, 2005).

1.2. Factors affecting viral survival in the marine environment

Viral survival is highly variable between virus types in the environment. There are several factors that control the survival of viruses in the marine environment, as water temperature, pH, exposure to UV, association with sediments, predators, presence of particular matter, salinity and raining (Le-Guyader *et al*. 1983, Chuan *et al*. 1983, Goyal *et al*. 1984, Yates *et al*. 1985, Griffin *et al*. 2003, Bosch *et al*. 2005; Bosch 2007; Fong *et al*. 2005, Bosch *et al*. 2006, Suttle 2007, Lugoli *et al*. 2009). These factors may act individually, or they may interact with each other, affecting viral survival in different ways (Cutler et al, 2012).

Water temperature

One of the most important factors controlling virus survival is water temperature (Yates *et al*. 1985, Bosch 2007; Bertrand et al, 2012), with surface water temperature playing a key role in the survival of enteric viruses in marine environments (Griffin *et al*. 2003).

Temperature affects the rate of protein and nucleic acid denaturation as well as the chemical reactions that can degrade the viral capsid and /or nucleic acids (e.g. enzymes), preventing adsorption of the viruses to their host and inactivating enzymes required for replication (Wetz *et al*. 2004, Fong *et al*. 2005, Bosch *et al*. 2006, Bosch 2007).

Several studies, have reported that, enteric viruses survive more frequently at lower temperature in natural environment (Fong *et al*. 2005, Bosch *et al*. 2006, Bosch 2007), being able to survive for many months at freezing or near-freezing temperatures (Bosch 2007). Enteric viruses can maintain their infectivity, even after long periods in the environment, surviving more than 130 days in seawater at temperatures between 20 and 30ºC (Rzezutka and Cook, 2004).

pH

Enteric viruses, in general, are very stable at the pH of most natural waters (pH 5 to 9) (Melnick *et al*. 1978, Duizer *et al*. 2004, Cannon *et al*. 2006, Bosch 2007), but most enteric viruses are more stable at low pH (3 to 5) (Melnick *et al*. 1978, Duizer *et al*. 2004, Thurston-Enriquez *et al*. 2005, Cannon *et al*. 2006, Bosch 2007), because the increase in pH has a direct toxic effect by fragmenting the nucleic acids (Bosch 2007, Abdel-Moety *et al*. 2008).

It has been suggested that the sensitivity of enteric viruses to pH may be strain dependent (Bosch 2007). Enterovirus can survive at very high pH (11.0 to 11.5) and very low pH (1.0 to 2.0) for short periods of time. Adenovirus and rotavirus are sensitive to inactivation at a pH of 10.0 or greater (Bosch 2007). Calicivirus are inactivated between pH 3 and pH 5 (Abdel-Moety *et al*. 2008). However, Duizer *et al*. (2004) shown that 3 h at pH 2.7 was not enough to completely inactivate norovirus.

UV light

The ultraviolet light of sunlight can inactivate viruses by causing cross-linking among the nucleotides (Suttle *et al*. 1992, Noble *et al*. 1997, Bosch 2007). Because UV radiation from the sun is present in the environment, natural defense mechanisms have evolved in microorganisms that allow UV inactivated microorganisms to reverse UV-induced damage through such repair pathways as photoreactivation and dark repair (Koivunen and Heinonen-Tanski, 2005; Quek and Hu, 2008; Hu et al, 2011). A microorganism UV resistance depends on the UV dose applied and its ability to protect itself from UV light and to repair damages (Sommer et al., 2001; Hu et al, 2011).

UV disinfection is highly effective against most viral pathogens at low doses (40 to 60 mJ/cm^2) commonly rendered in water treatment plants (Guo et al, 2010). However, viruses are more resistant to UV radiation than many other pathogens, because of their low molecular weight (Suttle *et al*. 1992, Bosch *et al*. 2006, Suttle, 2007).

The effectiveness of UV radiation in the inactivation of Calicivirus is comparable to that of the Enterovirus and less effective than that of Adenovirus (Thurston-Enriquez et al, 2003; Duizer *et al*. 2004).

Salinity

Although salinity does not present a direct effect on viral survival (Lo *et al*, 1976; Fong *et al*, 2005; Bosch *et al*, 2006), many studies relate that virus almost always survive longer in freshwater than in seawater (Wetz *et al*, 2004; Bosch, 2007; Seo et al, 2012). This may be due to the fact that high salinity concentrations are virucidal (Lo *et al*,

1976; Fong *et al*, 2005; Bosch *et al*, 2006) and because salinity increase viral aggregation, which appears to contribute to the loss of viral titre (Wetz *et al*, 2004; Bosch, 2007; Seo et al, 2012).

Adsorption to sediments

The association with sediments does not act only as reservoirs of human enteric viruses but also as a source from which viruses can be released into the water column by storm action and dredging activities (Rao *et al*, 1984; Bosch *et al*, 2005; Fong *et al*, 2005; Bosch, 2007). While viruses associated with small-size particulate material (<3 μm) tend to float in the water column, viruses adsorbed onto large/medium particles (>6 μm) readily settle down in the bottom sediment (Grabow, 1996; Bosch *et al*, 2005; Fong *et al*, 2005).

Adsorption of enteric viruses to sediments has been demonstrated to prolong the survival of Enterovirus (Bosch, 2007), which remain infectious for 19 days compared to 9 days for unassociated enteroviruses in the water column (Griffin *et al*,2003).

The increased virus survival in the presence of sediment has important implications in the marine environment, because fecal contamination of coastal areas results in contamination of shellfish harvesting areas, accumulation of solid-associated viruses in sediments, with sediments acting as virus reservoirs, and accumulation of viruses in shellfish (Bosch *et al*, 2006). Additionally, viruses concentration by molluskan bivalves is enhanced by the presence of particulate material (Bosch *et al*. 2006).

1.3. Enteric virus transmission from the marine environment

Viruses are a major cause of water-related disease (Bosch et al, 2008) and humans are exposed to marine enteric viruses through the consumption of shellfish grown in contaminated sea waters; to a lesser extend through sewage-polluted recreational waters, through the sea bathing and water sport activities, that are becoming increasingly popular and nowadays are expanded beyond the traditional summer seasons; and through marine aerosols (Bosch *et al*. 2005, Lipp *et al*. 2002, Bosch *et al*. 2006, Bosh *et al*. 2008).

Although much is known about the aquatic specific viruses that put in danger human populations, little is known about their natural reservoirs, namely the surface microlayer (SML), and their transmission to the atmosphere through aerosols.

Since many pathogenic viruses for humans circulate in the marine environment and some of them are considered emerging pathogens (Albinana-Gimenez et al., 2006;

Muscillo et al, 2008; LaRosa et al, 2012), it is pertinent to determine their density in SML, underlying water (UW) and atmosphere in order to assess the potential transmission via aerosols.

1.3.1. Role of SML in viral transmission

Aquatic surface microlayers are just a few tens of micrometers deep at the air–water interface and are physicochemically distinct from the UW below (Cunliffe et al, 2011). The primary interest in the structure and function of the SML is based on its crucial role in exchange processes of gases and matter across the air-water interface (Agouguè et al, 2004). The location of SML makes it a highly dynamic system. Atmospheric inputs to surface films include wet/dry deposition, air–sea gas and aerosol transfer. Exchange with the atmosphere is strongly influenced by the physicochemical and microbiological nature of SML (Cunliffe et al, 2011) (Figure 1.1).

Water column processes regulate the accumulation of material in the SML and small molecules and larger particles can accumulate at to form a film that extends into the UW (Cunliffe et al, 2011). In comparison with the UW, SML is physically more stable because of surface tension (Hardy, 1982) and the presence of a biogenic gelatinous film layer constitute a microbial habitat where compounds and particles can concentrate (Cunliffe et al, 2011). The SML is generally enriched in organic materials, which might stimulate biological growth. However, it has been shown that density, activity and diversity of microorganisms in the SML can be higher, similar or lower than those in UW. Unlike in the UW, microorganisms at SML receive maximal UV radiation, which has the potential to cause direct nucleic acid damage or indirect damage via the formation of destructive intermediates such as reactive oxygen species (Cunliffe et al, 2011). Organisms present in the SML may have developed strategies to survive in this habitat exposed to extreme conditions, and toxic heavy metals (Hardy 1982, Williams et al. 1986; Liss and Duce, 1997), but although these organisms may be exposed to the stress of SML, they can also be protected by the organic matrix present in this layer. SML habitats are unique as they interact with both the atmosphere and the hydrosphere simultaneously (Cunliffe et al, 2011).

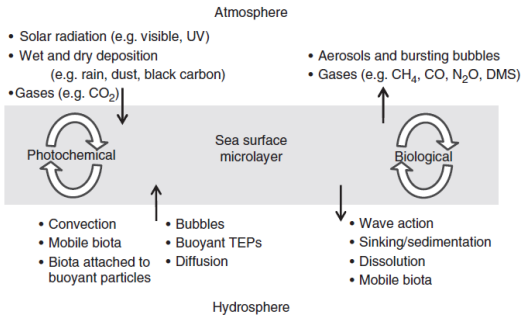

Figure 1.1: Inputs, outputs and processes whithin aquatic surface microlayer (Cunliffe et al, 2011).

Although viruses are the most abundant biological entity, representing the largest reservoir of genetic diversity, scarce information about their natural reservoirs, such as SML, is available. It is known that virus in SML are as abundant as in UW (Parada et al, 2005), but until now, there is no study on human pathogenic viruses in the SML. This can be, in part, due to difficulties in SML collection relatively to UW, which represents a greater problem for viral detection, since large volumes of water samples are needed.

The most commonly used devices for SML collection are mesh screens, often referred to as Garret screens (Garrett, 1965), glass plates (Harvey, 1965; Harvey & Burzell, 1972) and membranes (Crow et al., 1975; Kjelleberg et al., 1979), all of which whith inherent advantages and disadvantages (Cunliffe et al, 2011) (Figure 2).

Mesh screens rely on the collection of water in the interstitial spaces of a mesh. Metal or plastic mesh is stretched over and secured to a hand-held frame. The mesh screen is oriented horizontally and lowered through the SML into the UW, before being slowly withdrawn in the same way (Figure 2). Water retained within the mesh is then poured into a sampling vessel for processing as required (Cunliffe et al, 2011).

Glass plates (typically 20–30 cm^2) exploit the adhesion of a SML sample to a clean glass surface. Unlike the mesh, the glass plate is oriented vertically, lowered through the SML into the UW and slowly raised back out. The adhered sample is then removed using a wiper blade (e.g. neoprene) (Cunliffe et al, 2011) (Figure 1. 2).

Figure 1.2: Mesh screen and membrane surface microlayer samplers in use (Cunliffe et al, 2011), at left. Glass plates surface microlayer sampler in use, at right.

SML can also be sampled by adhesion to sterile membrane filters placed directly onto the water surface (Crow et al., 1975; Kjelleberg et al., 1979). Various types of membrane material are available; like polycarbonate and PTFE have been used (Crow et al, 1975; Kjelleberg et al, 1979; Cunliffe et al, 2011).Membranes present some distinct advantages over mesh screens and glass plates such the fact that they do not come into contact with the UW (Figure 2) (Cunliffe et al, 2011). The potential for contamination is virtually eliminated using forceps; individual membranes are lifted from the water surface with the SML attached and placed into storage vessels for processing.

1.3.2. Transmission of viruses by aerosols

Marine erosols are generally defined as colloidal systems of liquid or solid particles suspended in a gas (Hinds, 1999; Baron and Willeke, 2001; Fuzzi et al, 2006; Després et al, 2012), which can contain microorganisms and are generated through natural processes in the marine environment, like wave action (Fannin et al, 1985) or bubbles bursting through the SML (Aller et al, 2005; Morris et al, 2011). The size of aerosols can range from several nanometers to a few hundred micrometres in aerodynamic diameter (Cox and Wathes, 1995; Hinds, 1999; Jaenicke, 2005; Pöschl, 2005; Després et al, 2012) and viruses are transported in the smallest aerosols, with physical diameter as low as 20 nm (Dongsheng, 2006; Després et al, 2012). However, viruses are not commonly airborne as individuals and are more likely attached to other suspended particles (Després et al, 2012).

Marine aerosols can be classified as large respiratory droplets and droplet nuclei (Mubareka et al, 2009). Large respiratory droplets are > 5-15 μm in diameter and are involved in short-range transmission. Droplet nuclei are < 5 μm and are responsible for

transmission over great distances (long-range airborne transmission) (Mubareka et al, 2009).

Marine aerosols are formed primarily by the eruption of rising bubbles through SML (Aller et al, 2005; Morris et al, 2011) and contain high concentration of salts, organic matter, proteinaceous material, gel particles, microorganisms and viruses (Mathias-Maser & Jaenicke, 1994; Posfai, Li, Anderson & Buseck, 2003; St. Louis and Pelletier, 2004; Kuznetsova et al., 2005; Brooks et al, 2011; Morris et al, 2011; Danovaro et al, 2012). Bubbles eventually rise through the water column and upon reaching the sea surface, burst and eject aerosol droplets into the atmosphere, delivering the material carried by the bubbles to SML and to the atmosphere (Blanchard, 1975; Blanchard & Syzdek, 1982; Aller et al, 2005). Thus, the composition of marine aerosols formed from bursting bubbles at the sea surface changes in response to the SML composition (O'Dowd et al., 2004; Russell et al., 2010; Cunliffe et al, 2011). Bubble bursting and aerosol formation is an important transport mechanism for SML components (Russell et al., 2010). Bubbles generate the major portion of marine aerosols that are easily suspended and transported, through long distances, in the lower atmosphere (Gutafsson & Franzen, 2000; Grammatika & Zimmerman, 2001).

Aerosols formation constitutes the main vector for transport of viruses across the air-sea interface, playing an important role in long-distance dispersal and being responsible for the distribution of viruses (Mathias-Maser & Jaenicke, 1994; Posfai *et al*, 2003; St. Louis and Pelletier, 2004; Li et al, 2008; Brooks et al, 2011; Danovaro et al, 2012), since aerosols may remain suspended in the atmosphere for weeks (Dueñas, Fernandez, Carretero, Liger and Cñete, 2004). Any microorganism, and also viruses, can become airborne (Verrault et al, 2008). If the aerodynamic size of an infectious particle is appropriate, it can remain airborne, come into contact with humans or animals, and potentially cause an infection (Verrault et al, 2008). The probability of an airborne microorganism-laden particle causing an infection depends on its infectious potential and its ability to resist the stress of aerosolization (Verrault et al, 2008). Several studies have shown that marine aerosols large enough to contain organic particles, microbes and viruses can be transported for hundreds of kilometers from their source (Klassen and Roberge, 1999; Moorthy *et al*, 1998; Chow et al, 2000).

Aerosols can be single virus, aggregates of one or several types of particles, or attached to non-biological particles (Sun and Ariya, 2006; Morris et al, 2011). While single virus particles exist in the air, they tend to aggregate rapidly (Verrault et al, 2008), with aggregation speed depending on the size distribution of the airborne particles, the concentration of the aerosol, and of the thermodynamic conditions (142;

(Verrault et al, 2008). Organic and inorganic materials in viral aerosols can affect the size of the aerosolized particles and their infectious potential. Many factors, such as relative humidity, temperature, radiation, aerosolization medium, exposure period, chemical composition of the air and also the sampling methods, can affect the infectivity of airborne viruses (Verrault et al, 2008).

When a virus is contained in an aerosol, their infectivity is increased, compared to a virus free in the air, since the aerosol prevent desiccation and protects from temperature and sunlight (Li et al, 2008) and so, aerosols of pathogenic viruses pose an increased threat to health (Adams et al, 1982). For the past 200 years, the field of aerobiology has explored the abundance, diversity, survival and transport of microorganisms in the atmosphere (Morris et al, 2011). Yet, the atmosphere still presents a frontier for pioneering microbiologists (Verrault et al, 2008; Morris et al, 2011). This is due mainly to the difficulty in collecting and analyzing airborne biological contaminants, which is an even greater problem for viral detection (Verrault et al, 2008). The perceived lack of research might also be connected to the fact that before the development of molecular biological methods (e.g. PCR) to detect genetic material of microorganisms (Alvarez et al., 1995; Peccia and Hernandez, 2006), only viable viruses could be found in air samples (Morris et al, 2011). Another possible reason for the scarcity of publications containing information on viable viruses in atmospheric aerosol is inactivation of viruses in the atmosphere under the influence of different environmental factors (changes in temperature, relative humidity, solar radiation, etc.). Aerosols sampling techniques have been improved greatly over the years, giving more ability to tackle the important health issue of airborne viruses. However, the lack of standardization has to be addressed, as it limits the development of general recommendations for sampling of airborne viruses (Verrault et al, 2008). Studies to date have rarely included quantitative analyses of total viral load. As far as the knowledge of viruses in the aerosol is concerned, most of the studies have been focused on the analysis of specific pathogenic viruses and their infectivity (Ijaz et al, 1987; Sagripanti and Lytle, 2007). Only two studies on marine aerosol have reported information on the abundance of viruses and their enrichment factor in comparison with the SML (Aller et al, 2005; Kuznetsova et al, 2005).

The probability of detecting airborne viral pathogens is dependent on three primary factors: the concentration of airborne viruses in the environment, the ability of the air-sampling system to recover airborne particles (collection efficiency), and the analytical sensitivity of the diagnostic assay(s) used to detect the target pathogen in the sample (Herman et al, 2006; Verrault et al, 2008).

The sampling and collection of atmospheric aerosols requires a great understanding of the physical principles that govern interactions with suspended particles. As such, particle size is by far the most important characteristic for choosing a sampling procedure for airborne particles (Nicholson, 1995; Després et al, 2012). A representative sample should contain nanoparticles with larger particles (Verrault et al, 2008). However, the abiotic factors are also important to evaluate the possible formation of aerosol and the subsequent transmission of viruses from SML to the atmosphere. It is known that different environmental conditions, including relative humidity, temperature, and UV radiation, wind velocity and particulate matter, influence the characteristics of the viruses, including concentration and infectivity (Li et al, 2008).

Collection of living, or viable, material requires care in order not only to sample microorganisms appropriately but also to keep them alive until the desired analysis can be performed (Després et al, 2012).

Different methods have been developed based on the attachment properties to surfaces of airborne particles. The most used air samplers are based on impact on solid surfaces, impingement, and filtration (Figure 3). All of them have been successfully used for virus detection, but have advantages and disadvantages (Verrault et al, 2008; Bosch et al, 2011).

While culture is often used to determine viral concentrations and most sampling methods affect viral infectivity, culture methods become inadequate for calculating the true concentrations of infectious airborne viruses. Technologies such as PCR can be used to detect viruses in air samples even when they are no longer infectious (Verrault et al, 2008).

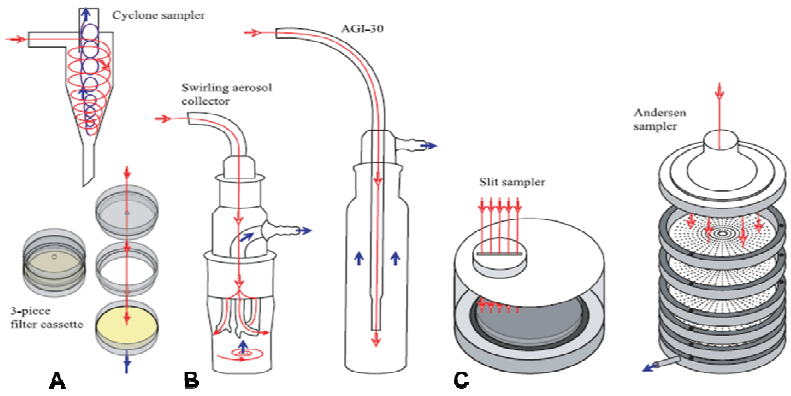

Figure 1.3: Different bioaresol samplers (Verrault et al, 2008). **A**: Impact samplers; **B**: Impinge samplers and **C**: Filter samplers.

Aerosols collection by impact samplers or solid impactors

The impact samplers are usually more efficient at capturing large particles. Andersen and slit samplers accelerate the particles through narrow holes or slits. The streamline moves toward a solid surface and abruptly changes direction (Figure 1.3.A). The inertia of the particles deviate them from the airflow and impacts them on the surface, which usually holds a petri dish with a culture medium. The medium is either washed to collect the particles or used directly for plaque assays (Verrault et al, 2008).

Slit samplers are used mostly to determine aerosol concentrations of microorganisms as a function of time. The accelerated particles are impacted onto a rotating petri dish containing a culture medium with a liquid layer, making it possible to determine the time when each particle was sampled (Verrault et al, 2008).

Impact samplers are easy to use, but dehydratation or impact trauma can affect viral survival. Flow rate and sampling duration are crucial when using these samplers (Bosch et al, 2011).

Aerosols collection by impinge samplers or liquid impactors

The all-glass impingers (AGI) are the most often used samplers for the capture of airborne viruses (Verrault et al, 2008). The liquid impinge offer the possibility of extended sampling times (Després et al, 2012) and it works by accelerating airborne particles through a narrow orifice placed at a fixed distance from the bottom of a flask containing a liquid. A pressure drop is created in the flask and forces the air to enter through the inlet of the impinger. The air enters horizontally through a glass tube, which curves to a vertical position, forcing the air to change direction and flow downward. The diameter of the tubing abruptly narrows and acts as a critical flow orifice, accelerating the air passing through it to sonic velocity. The curve in the tube is intended to trap the larger particles by inertial impaction and mimics the airway of the human nose (Herman et al, 2006; Verrault et al, 2008) (Figure 1.3.B). The formation of small bubbles in the liquid of the impinger can also help to sample very small particles by diffusion. However, the reaerosolization of particles due to the scavenging properties of the air bubbles can be a problem, especially for hydrophobic particles (Verrault et al, 2008). After sampling, the medium can be concentrated or directly decontaminated, purified, and analyzed (Bosch et al, 2011). The recovery efficiency of this method is high because it avoids dehydration and the liquid facilitates the extraction of genetic material, for subsequent analysis (Verrault et al, 2008; Bosch et al, 2011). However, flow rate and the composition of the collection fluid are critical for virus recovery (Bosch et al, 2011).The gentler sampling process leading to better recovery of infective viruses

seems to be the main reason for the wide use of AGI samplers in aerovirology (Verrault et al, 2008).

A number of variables are known to affect impinger collection efficiency. These include impinger design (Cage et al, 1996; Donaldson et al, 1982; Herman et al, 2006; Jensen et al, 1992), sampling time (Lin and Li, 1998), and the composition of collection medium (Lin et al, 2000). Sampling times reported for the recovery of airborne pathogens using air impingers are variable, ranging from minutes to hours (Terzieva et al, 1996; Donaldson et al, 1987; Lin and Li, 1998; Lin and Li, 1999; Herman et al, 2006). Likewise, flow rates described for the collection of airborne viruses are variable, ranging from 12.5 liters/min (Elazhary and Derbyshire, 1979) to 450 liters/min (Dee et al, 2005; Herman et al, 2006). Collection media described for the recovery of airborne pathogens in air impingers are diverse including deionized water (Lin et al, 1997), buffered solutions (Juozaitis et al, 1994) and mineral oil (Lin et al, 2000). Compounds added to collection medium to improve pathogen recovery include various proteins (Stolze et al, 1989) and antifoaming agents (Karim et al, 1985; Schoenbaum et al, 1990; Chinivasagam and Blackall, 2005; Herman et al, 2006). According to Harstad (1965), liquid impingers are the least destructive samplers, being 18% more efficient, although 30% to 48% of the sample is physically lost (Després et al, 2012; Herman et al, 2006; Verrault et al, 2008).

Aerossols collection by filter samplers

In filter samplers, the air passes through a filter and airborne particles are retained (Bosch et al, 2011) (Figure 1.3.C). Filter efficiency is based on the following five basic mechanisms: (i) interception, (ii) inertial impaction, (iii) diffusion, (iv) gravitational settling and (v) electrostatic attraction (Hinds, 1999). Filtration efficiency improves with increasing and decreasing particle size (Verrault et al, 2008).

Many different types of filters have been used to sample airborne viruses. They differ mainly in composition, pore size, and thickness (tightly packed cotton, cellulose filters (0.45μm pore size), PTFE filters (2.0μm pore size) and gelatin filters) (Verrault et al, 2008; Després et al, 2012). Polycarbonate filters are much less efficient than gelatin or PTFE filters (Burton et al, 2007). The low filtration efficiency of polycarbonate filters may be due to the structure of the filter. The contact area of filters with uniform cylindrical pores, such as polycarbonate filters, is much smaller than that of filters with a complex structure, such as PTFE filters, where the probability of adherence is greater because airborne particles are exposed to a greater surface area (Verrault et al, 2008).

Gelatin filters can be used because they do not appear to significantly affect viral infectivity, but they can be limited by environmental conditions. Low humidity can cause them to dry out and break, while high humidity or water droplets can cause them to dissolve (Bosch et al, 2011; Verrault et al, 2008).

Despite this sampling method is easy to use, filters are not commonly used to sample airborne viruses because they can cause structural damage (Verrault et al, 2008). In addition, the desiccation of the samples that occurs during sampling can interfere with culture analysis of the samples (Bosch et al, 2011; Verrault et al, 2008). With this sampling method, flow rate, the sapling duration and the membrane composition have to be strictly controlled to avoid dehydration (Bosch et al, 2011).

1.4. Detection of enteric viruses in the aquatic system

1.4.1. Viral concentration methods

Although viruses are very abundant in the aquatic systems, the number of viruses relevant to public health, in this environment is low (Miki and Jacquet, 2008). However, even pathogenic viruses have low infectious doses, even at low concentrations, these viruses can cause diseases. Therefore, direct viral detection is impossible and it is necessary to concentrate large volumes of water to only a few milliliters, before proceeding to the detection (Bosch et al, 2008). Sometimes it is necessary a two-step concentration procedure, with polyethyleneglycol precipitation (PEG) and ultrafiltration as preferred procedures for reconcentration of the primary eluates (Bosch et al, 2008).

A good concentration method should fulfill several criteria, it should be technically simple, be fast, provide high viral recovery, be adequate for a wide range of virus and samples type, provide a small volume of concentrate, be cost effective (Bosch, 1998) and should not interfere with viral community structure (Angly et al, 2006). A variety of strategies have been used to concentrate viruses from water samples, based mostly on flocculation, adsorption–elution, ultrafiltration and ultracentrifugation techniques. However, there is not yet a single method that enables a highly efficient concentration of all viruses (Block and Schwartzbrod, 1989; Bosch *et al*, 2008, Albinana-Gimenez *et al*, 2009).

Different types of filters and filtration methods, such as cartridge filters (electropositive or electronegative), glass fiber filters, glass wool filters, vortex flow filtration, tangential flow filtration and acid flocculation, have been used to collect and concentrate viral particles from water samples. Because of the small size of viral particles, mechanical filtration is often not possible and therefore, adsorption- elution methods are employed.

Adsorption-elution and flocculation methods are most frequently used to concentrate viruses in environmental waters because they allow to process large volumes of water (Katzenelson *et al*., 1976; Puig *et al*., 1994; Calgua *et al*., 2008) relatively to ultrafiltration method, wich has a slow filtration rate (Muscillo *et al*., 2008, Kovac *et al*., 2009; Cashdollar & Wymer, 2013). However, for these methods the electrostatic interaction between the virus and the surface of the filters/flakes depends on various factors, including viral isoelectric points, water pH, and salt concentration (Hsu *et al*, 2007; Polaczyk *et al*, 2007; Victoria *et al*, 2009), which imply water sample manipulation. Ultrafiltration methods are an alternative to adsorption-elution and flocculation techniques and have shown to be efficient to recover viruses from marine water, since they require minimal manipulation. Samples can be processed under natural pH and an elution step is not needed (Fong and Lipp, 2005). Ultracentrifugation is a good alternative method for viral concentration in environmental water samples because it does not modify the viral community present in the sample (Fumian et al, 2010; Prata et al, 2012).

The comparison among different concentration methods is difficult because it is necessary to take into account many variables (e.g. type of sample, volume of water and the methods used to determinate the recovery efficiency). Nevertheless, in general, for environmental waters, the ultracentrifugation method allows viruses recovery efficiencies similar to those of adsorption–elution and flocculation methods (Guttman-Bass and Armon, 1983; Shields and Farrah, 1986; Puig et al, 1994; Calgua et al, 2008).

Flocculation

Flocculation methods are the most frequently used to concentrate viruses in environmental waters because they allow to process large volumes of water (Calgua et al, 2008; Katzenelson et al, 1976; Puig et al, 1994) relative to ultrafiltration methods (Muscillo et al, 2008, Kovac et al, 2009). In general, a buffered beef extract is used to precipitate viruses from samples by reducing the pH to 3.5, after which samples are centrifuged and the viral pellet ressuspended in tris-buffered saline (Fong and Lipp, 2005). The precipitate is then centrifuged to form a pellet before being dissolved in sodium phosphate (Enriquez et al, 1995; Fong and Lipp, 2005). The polyethylene glycol (PEG) precipitation procedure consists of precipitating viral particles by addition of 0.5 M NaCl and 7% PEG to beef extract with constant stirring for 2 h at 4°C followed by centrifugation. The viral pellet is then resuspended in tris-buffered saline (Enriquez et al, 1995; Fong and Lipp, 2005). The use of beef extract in these procedures has

been reported to cause inhibitory effects in PCR assays (Arnal et al, 1999; Schwab et al, 1995; Fong and Lipp, 2005).

Adsorption-elution

Some of the earliest methods in environmental virology involved adsorption of the viral particle to a surface and elution from that surface (Cashdollar and Wymer, 2013). These methods are used as an alternative to filtration methods and involve manipulation of charges on the virus surface, using pH changes to maximize their adsorption to charged filters (Fong and Lipp, 2005; Cashdollar and Wymer, 2013).

Enteric viruses have considerable variation in the number of proteins present in their capsid, which then affects size and charge of the proteins that make up the capsid.

Enteric viruses range from about 30 nm (enterovirus) to 100 nm (adenovirus) in diameter (Michen and Graule 2010). Because viruses in water typically have a net negative surface charge, depending on the type of filter used, either the filters or the water sample has to be conditioned prior to filtration of the sample to allow adsorption. There are two basic filter types used to adsorb virus: electronegative filters and electropositive filters (Cashdollar and Wymer, 2013).

Electropositive filters require no manipulation of pH because most enteric viruses are negatively charged at ambient pH (Lipp et al, 2001; Fong and Lipp, 2005). However, electropositive filters are easily clogged and have low recovery rates for viruses in marine water; since the presence of salt and alkalinity of seawater cause low adsorption of viruses to the filters (Lukasik et al, 2000; Fong and Lipp, 2005).

Electronegative filters show higher virus recoveries from marine water and waters of high turbidity than do electropositive filters (Enriquez et al, 1995, Lukasik et al, 2000; Lipp et al, 2001, Katayama et al, 2002; Fong and Lipp, 2005). Since under ambient conditions, enteric viruses are negatively charged they only adsorb to a negatively charged membrane in the presence of Mg^{2+}, other multivalent cations, or, more commonly, under acidic conditions (Fong and Lipp, 2005).

Ultrafiltration

Ultrafiltration has gained popularity in the last decade as an attractive method for virus concentration from large volumes of water (Cashdollar and Wymer, 2013). This method relies on size exclusion and because of the pore size, water and low molecular weight substances are allowed to pass through the fibres and into the filtrate, whereas larger substances, such as viruses and microrganisms, are trapped and retained in the retentate (Cashdollar and Wymer, 2013).

Different types of filters (electropositive or electronegative; glass fibre; glass wool filters) and ultrafiltration methods (vortex flow filtration; tangential flow filtration) can be used to concentrate viral particles in water samples (Fong and Lipp 2005). These methods require minimal manipulation of water and samples can be processed at natural pH with no elution step being needed (Fong and Lipp, 2005).

Ultrafiltration methods such as vortex flow filtration (VFF) and tangential flow filtration (TFF) are alternatives to adsorption- elution techniques and have been shown to be efficient in recovering viruses from marine water (Paul et al, 1991; Griffin et al, 2003; Fong and Lipp, 2005). Both filtration devices use a flow pattern that forces water through a cylindrical filter with pressure while keeping and retaining particles from filters to avoid clogging (Paul et al, 1991; Fong and Lipp, 2005). TFF requires prefiltration of water samples to remove plankton and suspended solids. VFF has been shown to be more time-efficient because prefiltration of samples is not required, and it has a higher viral recovery rate than TFF, but it tends to concentrate more PCR inhibitors with the virus (Jiang et al, 2001; Fong and Lipp, 2005). However, both VFF and TFF are less cost- and time-effective than adsorption-elution because of the high cost of equipment and limitations on the volume of sample that can be concentrated at one time (Fong and Lipp, 2005).

Ultracentrifugation

Ultracentrifugation is a good alternative method for viral concentration in environmental water samples since it is simple, requires minimal manipulation, samples can be processed under natural pH and an elution step is not needed (Percival et al, 2004; Fumian et al, 2010). By ultracentrifugation it is possible to concentrate all viruses in a sample, by using a sufficient g-force during an adequate period of time (Percival et al, 2004) and it does not introduce any PCR inhibitory substance and, consequently, concentrated samples can be successfully used for molecular detection (Fumian et al, 2010). Although it is difficult to process large volumes of water with this method it is possible to reduce the volume of the sample to fewer millilitres than with adsorption–elution and flocculation methods (Fong and Lipp, 2005). Another advantage of this method is that it has higher recovery rate. However, this recovery efficiency depends on the physical and chemical properties of each virus, including specific density, morphology and membrane attachment patterns (Fumian et al, 2010).

1.4.2. Identification and quantification methods

Concentrated samples can be either extracted for viral nucleic acid analysis (PCR), inoculated onto common cell culture lines or used with immunological methods, for enteric viruses detection (Fong and Lipp, 2005; Bosch et al, 2011).

Traditional assays

Before the development of molecular methods, enteric viruses diversity, identity and abundance were primarily studied via cultivation methods (Després et al, 2012; Mojica et al, 2014). Besides the cultivation methods, there are electron microscopy and epifluorescence microscopy in combination with nucleic acid-specific staining and immunological methods (such as immunohistochemical detection, indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA)), that allow to detect enteric viruses, (Piao et al, 2012; Mojica et al, 2014). However, these methods are limited by either the availability of a host system, cost, or time (Piao et al, 2012; Gentile & Gelderblom, 2014; Mojica et al, 2014).

Molecular assays

Molecular techniques always start with viral nucleic acid extraction from the sample. There are several methods that can be used for viral detection on environmental samples, such as PCR; multiplex PCR; real time PCR, pyrosequencing and integrated cell culture PCR. (Fong and Lipp, 2005). Molecular techniques have been used extensively to detect enteric viruses from environmental samples since the early 1990s and usually are based on the detection of a part of the viral genome that is highly conserved with broad homology within a specific group of virus (Fong and Lipp, 2005). PCR-based techniques offer several advantages over cell culture assays in detecting viral pathogens from environmental samples, because are less time consuming and laborious and can be highly sensitive and specific if a well-designed assay is developed (Fong and Lipp, 2005).

PCR

The PCR technique efficiently amplifies characteristic regions of the DNA of a specie, or a group of species, for detailed analyses (Fong and Lipp, 2005; Després et al, 2012).

Results from PCR assays can be obtained within 24 h of sampling, compared to days or weeks of incubation for cell culture assay (Griffin et al, 1999; Noble et al, 2003; Fong and Lipp, 2005). PCR technique is also highly sensitive and is capable of detecting

virus that are present in low numbers in environmental samples and or that are difficult to grow in cultured cells (Pommepuy and Le Guyader, 1998; Chapron et al, 2000; Lipp et al, 2001; Fong and Lipp, 2005). Theoretically, the DNA polymerase can amplify a single DNA copy and thus detect even organisms that are present in minor quantities (Després et al, 2012). But, the sensitivity of the PCR depends on several factors, such as the primer pair used for the amplification process (different primer pairs have different specificity and sensitivity (Alvarez et al, 1995; Polz and Cavanaugh, 1998; Després et al, 2012), the possible presence of PCR inhibitors and the integrity of the DNA molecule (Després et al, 2012).

The high level of sensitivity in PCR assays has indicated that cell culture detection alone may underestimate the true level of contamination in environmental sources (Fong and Lipp, 2005). Unlike with cell culture, however, the infectivity of viruses detected by molecular methods is often unknown.

False-negative results may also be a problem when inhibitors in environmental samples are present. Humic and fulvic acids, heavy metals, and phenolic compounds may inhibit the activity of polymerase enzyme (Young et al, 1993; Straub et al, 1995; Wilson, 1997; Fong and Lipp, 2005), either by hindering the attachment of polymerase to the primers to initiate amplification, or by binding to the DNA and thereby preventing primers or enzymes from attaching (Després et al, 2012).

Some variations of conventional PCR include nested PCR, multiplex PCR, and real-time PCR (for quantification). Seminested PCR and nested PCR assays increase the sensitivity and specificity of PCR with the use of an internal primer or primer set and are sometimes used as a confirmation step (Fong and Lipp, 2005).

Multiplex PCR

The application of multiplex PCR (where several sets of primers against several targets are included in a single PCR) may save time and costs because several types of virus can be detected in a single PCR assay (Fout et al, 2003; Fong and Lipp, 2005). The development of a multiplex PCR assay, however, is not easy and requires careful optimization of reaction mixtures and PCR conditions (Green et al, 1999; Tsai et al, 1993; Fout et al, 2003; Fong and Lipp, 2005).

Real time-PCR

Real-time PCR (RT-PCR) provides quantitative data for the presence of enteric viral genomes in environmental samples with the use of a fluorescent dye, such as SYBR Green, that will bind to amplified nucleic acid or with fluorochrome-tagged probes that

fluoresce when bound to complementary sequences in the amplified region (Fong and Lipp, 2005). The procedure is less time-consuming because a confirmation step such as agarose gel electrophoresis and additional hybridization are generally not required. The entire analysis can be done in a closed system, which may reduce the potential for contamination (Fong andLipp, 2005). However, the cost of a real-time PCR instrument is still substantially more than that of a conventional PCR instrument, and in some cases, real-time PCR has been shown to be less sensitive than conventional RT-PCR and nested PCR (Noble et al, 2003; Fong and Lipp, 2005).

Pyrosequencing

To determine the identity of the genomes obtained, the PCR products are often cloned and sequenced (Boreson et al, 2004; Maron et al, 2005; Després et al, 2007; Fierer et al., 2008; Bowers et al., 2009; Fröhlich-Nowoisky et al, 2009; Georgakopoulos et al., 2009; Després et al, 2012). Pyrosequencing uses luciferase to generate light for detection of the individual nucleotides added to the nascent DNA, and the combined data are used to generate sequence readouts (Després et al, 2012). Species can often be identified by comparing the obtained sequences with those that are already available in online databases like the National Center for Biotechnology Information (NCBI) (Després et al, 2012; Zao et al, 2013).

The high demand for low-cost sequencing in recent years has led to the development of high-throughput sequencing technologies. In these technologies, the sequencing process is parallelized for several samples, and thus in a short time thousands or millions of sequences are produced (Després et al, 2012). The advent of whole-community genome sequencing is rapidly changing the way viral and microbial diversity are assayed. Using this approach, it is possible to rapidly characterize the metabolic diversity and community structure of any microbial ecosystem (Angly et al, 2006). Moreover, quick and accurate identification of microbial pathogens is essential for both diagnosis and response to emerging infectious diseases. High-throughput sequencing has recently emerged as a powerful approach to identify both known and novel viruses in clinical specimens (Zao et al, 2013).

Integrated cell culture PCR (ICC-PCR)

While PCR-based methods offer many advantages in sensitivity, specificity, and efficiency over cell culture, they still cannot provide information on the infectivity of viruses detected with the reliability of cell culture. (Fong and Lipp, 2005). Cell culture combined with PCR (ICC-PCR) is an approach that has been used to overcome most

of the disadvantages associated with both conventional cell culture and direct PCR assays (Reynolds, 2004; Rodríguez et al, 2009). Detection relies on an initial biological amplification of viral nucleic acid, followed by amplification via PCR (Reynolds et al, 1994; Rodríguez et al, 2009). Viruses are allowed to replicate in cell culture for short periods followed by PCR amplification, which dramatically reduces the time necessary for infectious viral detection (Reynolds, 2004; Rodríguez et al, 2009). ICC-PCR has also the advantage of detecting viable viruses that do not produce cytophatic effects (CPE). The sensitivity obtained with ICC-PCR is comparable to that obtained in cell culture after a second passage in cell culture (Blackmer et al, 2000; Rodríguez et al, 2009). In addition, fewer problems are encountered with inhibitory compounds that may be contained in environmental concentrates (Chapron et al, 2000; Rodríguez et al, 2009).

The use of ICC-PCR has been described for the detection of enteroviruses (Reynolds et al, 1996; Rodríguez et al, 2009), hepatitis A virus (Reynolds et al, 2001;Jiang et al, 2004; Rodríguez et al, 2009), enteric adenoviruses (Lee and Kim, 2002; Rodríguez et al, 2009), and astroviruses (Reynolds et al, 2001; Rodríguez et al, 2009).

References

- Abdel-Moety N M, Al-Fassi F A, Ali M A, 2008, Health aspects of virological water quality: an overview review, Journal of Applied Sciences Research, 4 (10), 1205-1215.
- Acosta B, Odalys V, Alexander P, Clara S, Angel G, 2009, Molecular characterization of adenoviral infections in Cuba: report of an unusual association of species D adenoviruses with different clinical syndromes, Archives of Virology, 154: 619-627.
- Adams D J., Spendlove J C., Spendlove R S, Barnett B B, 1982, Aerosol Stability of Infectious and Potentially Infectious Reovirus Particles, Applied and Environmental Microbiology, 44 (4): 903-908.
- Agogué H, Casamayor E O, Joux F, Obernosterer I, Dupuy C, Lantoine F, Catala P, Weinbauer M G, Reinthaler T, Herndl G J, Lebaron P, 2004, Comparison of samplers for the biological characterization of the sea surface microlayer, Limnology and Oceanography Methods, 2, 213–225.
- Albinana-Gimenez N, Clemente-Casares P, Bofill-Mas S, Hundesa A, Ribas F, Girones R, 2006, Distribution of human polyomaviruses, Adenoviruses and hepatitis E virus in the environmental and drinking-water treatment plant, Environmental Science and Technology, 40, 7416–7422.
- Albinana-Gimenez N, Miagostovich M P, Calgua B, Huguet J M, Matia L, Girones R, 2009, Analysis of Adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants, Water Research, 43, 2011–2019.
- Allard A, Albisson B, Wadell G, 2001, Rapid Typing of Human Adenoviruses by a General PCR combined with Restriction Endonuclease Analysis, Journal of Clinical Microbiology, 39 (2) 498–505
- Aller J Y, Kuznetsova M R, Jahns C J, Kemp P F, 2005, The sea surface microlayer as a source of viral and bacterial enrichment in marine aerosols, Aerosol Science, 36, 801–812
- Alvarez A J, Buttner M P, Stetzenbach L D, 1995, PCR for bioaerosol monitoring: sensitivity and environmental interference, Applied and Environmental Microbiology, 61 (10), 3639-3644.
- Andrady A, Aucamp P J, Bais A F, Ballare C L, Björn L O, Bornman J F, Caldwell M, Cullen A P, Erickson D J, deGruijl F R, Häder D P, Ilyas M, Kulandaivelu G, Kumar H D, Longstreth J, McKenzie R.L, Norval M, Paul N, Redhwi H H, Smith R C,

Solomon K R, Sulzberger B, Takizawa Y, Tang X, Teramura A H, Torikai A, van der Leun J C, Wilson S R, Worrest R C, Zepp R G, 2010, Environmental effects of ozone depletion and its interactions with climate change: progress report, 2009. Photochemistry and Photobiology Science, 9, 275–294.

- Angly F E, Felts B, Breitbart M, Salamon P, Edwards R A, Chan A M, Haynes M, Kelley S, Liu H, Mahaffy J M, Carlson C, Mueller J E, Nulton J, Olson R, Parsons R, Rayhawk S, Suttle C A, Rohwer F, 2006, The Marine Viromes of Four Oceanic Regions, PLoS Biol, 4(11: e368.
- Aragão G C, Oliveira D S, Santos M C, 2010, Molecular characterization of norovirus, sapovirus and astrovirus in children with acute gastroenteritis from Belém, Pará, Brazil, Rev Pan-Amaz Saude, 1(1) 149-158.
- Arnal C, Ferre-Aubineau V, Besse B, Mignotte B, Schwartzbrod L, Billaudel S, 1999, Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A amplification, Journal of Virological Methods, 77, 17-26.
- Asanaka M, Atmar R L, Ruvolo V, 2005, Replication and packaging of Norwalk virus RNA in cultured mammalian cells, PNAS, 102 (29).
- Aslan A, Xagoraraki I, Simmons F J, Rose J B, Dorevitch S, 2011, Occurrence of adenovirus and other enteric viruses in limited-contact freshwater recreational areas and bathing waters, Journal of Applied Microbiology, 111, 1250–1261.
- Attoui H, Jaafar F M, Belhouchet M, Biagini P, Cantaloube J-F, Micco P, Lamballerie X, 2005, Expansion of family Reoviridae to include nine-segmented dsRNA viruses: Isolation and characterization of a new virus designated aedes pseudoscutellaris reovirus assigned to a proposed genus (Dinovernavirus), Virology, 343, 212 – 223.
- Attoui H, Jaafar F M, Belhouchet M, Micco P, Lamballerie X, Brussaard C, 2006, Micromonas pusilla reovirus: a new member of the amily Reoviridae assigned to a novel proposed genus (Mimoreovirus), Journal of General Virology, 87, 1375–1383.
- Barlan A U, Griffin T M, McGuire K A, Wiethoff CM, 2011, Adenovirus membrane penetration activates the NLRP3 inflammasome, Journal of Virology, 85, 146155.
- Baron P A, Willeke K, 2001, Gas and Particle Motion, In Aerosol Mea-surement: Principles, Techniques and Applications, Wiley., New York. pp. 6 1-97.
- Bertrand I, Schijven J F, Sanchez G, Wyn-Jones P, Ottoson J, Morin T, Muscillo M, Verani M, Nasser A, Husman A R R, Myrmel M, Sellwood J, Cook N, Ganzer C, 2012, The impact of temperature on the inactivation of enteric viruses in food and water: a review, Journal of Applied Microbiology, 112, 1059–1074.

- Bigoraj E, Kwit E, Chrobocinska M, Rzezutka A, 2014, Occurrence of Norovirus and Hepatitis A Virus in Wild Mussels Collected from the Baltic Sea, Food Environ Virol, DOI 10.1007/s12560-014-9153-5.
- Blackmer F, Reynolds K A, Gerba C P, Pepper I L, 2000, Use of integrated cell culture-PCR to evaluate the effectiveness of poliovirus inactivation by chlorine, Applied and Environmental Microbiology, 66, 2267–2268.
- Blanchard D C, 1975, Bubble scavenging and water-to-air transfer of organic material in sea, Advances in Chemistry Series, 145, 360–387.
- Blanchard D C, Syzdek L, 1982, Water-to-Air Transfer and Enrichment of Bacteria in Drops from Bursting Bubbles, Applied and Environmental Microbiology, 43 (5) 1001-1005.
- Block J. C., Schwartzbrod L., 1989, Viruses in Water Systems: Detection and Identification, VCH Publishers, Inc, New York.
- Bofill-Mas S, Calgua B, Clemente-Casares P, La Rosa G, Iaconelli M, Muscillo M, Rutjes S, de Roda Husman A M, Grunert A, Gräber I, Verani M, Carducci A, Calvo M, Wyn-Jones P, Girones R, 2010, Quantification of Human Adenoviruses in European Recreational Waters, Food and Environmental Virology, 2 (2) 101-109
- Boreson J, Dillner A, Peccia J, 2004, Correlating bioaerosol load with PM2.5 and PM10cf concentrations: a comparison between natural desert and urban-fringe aerosols, Atmospheric Environment, 38, 6029-6041.
- Bos T J, Somers K D, 2004, USMLE Road Map: Microbiology & Infectious Disease, McGraw-Hill Professional.
- Bosch A, 1998, Human enteric viruses in the water environment: a minireview, Int. Microbiology, 1, 191–196.
- Bosch A, Abad F X, Pintó R M, 2005, Human Pathogenic Viruses in the Marine Environment, Oceans and Health: Pathogens in the Marine Environment, 109-131
- Bosch A, Abad F X, Pintó R M, 2006, Survival and transport of enteric viruses in the environment, In “Viruses in Food”, S. M. Goyal (Ed.), Food Microbiology and Food Safety Series, Springer, New York, pp 151-187.
- Bosch A, 2007, Viruses Ocurrence and Survival in the Environmental waters, In “Human Viruses in water”, First Edition, Elsevier, pp 91-108.
- Bosch A, Guix S, Sano D, Pinto R M, 2008, New tools for the study and direct surveillance of viral pathogens in water, Current Opinion in Biotechnology, 19, 295–301.

- Bosch A, Sánchez G, Abbaszadegan M, Carducci A, Guix S, Le Guyader F S, Netshikweta R, Pintó R M, van der Poel W H M, Rutjes S, Sano D, Taylor M B, van Zyl W B, Rodríguez-Lázaro D, Kovač K, Sellwood J, 2011, Analytical Methods for Virus Detection in Water and Food, Food Analytical Methods,4 (1), 4-12.
- Bowers R M, Lauber C L, Wiedinmyer C, Hamady M, Hallar A G, 2009, Characterization of airborne microbial communities at a high-elevation site and their potential to act as atmospheric ice nuclei, Applied and Environmental Microbiology, 75, 5121-5130.
- Brooks I M, Andreas E L, McFIiggans G, Anguelova M D, O'Dowd C, 2011, Primary Marine Aerosol Fluxes: Progress and Priorities, American Meteorological Society, 489-491.
- Burton N C, Grinshpun S A, Reponen T, 2007, Physical Collection Efficiency of Filter Materials for Bacteria and Viruses, Ann. Occup. Hyg, 51 (2) 143–151.
- Caballero S, Abad F X, Loisy F, Le Guyader F S., Cohen J, Pintó R M, Bosch A, 2004, Rotavirus Virus-Like Particles as Surrogates in Environmental Persistence and Inactivation Studies, Applied and Environmental Microbiology, 70 (7) 3904-3909.
- Cage B R, Schreiber K, Barnes C, Portnoy J, 1996, Evaluation of four bioaerosol samplers in the outdoor environment, Ann. Allergy Asthma Immunology, 77, 401–406.
- Calgua B, Mengewein A, Grunert A, Bofill-Mas S, Clemente- Casares P, Hundesa A, Wyn-Jones A P, Lopez-Pila J M, Girones R, 2008, Development and application of a one-step low cost procedure to concentrate viruses from seawater samples, Journal of Virological Methods, 153, 79–83.
- Cannon J L, Park G W, Osborne J, Jaykus L A, Vinje J, 2006, Surrogates for the Study of Norovirus Stability and Inactivation in the Environment: A Comparison of Murine Norovirus and Feline Calicivirus, Journal of Food Protection, 69(11) 2761–2765.
- Cashdollar J L, Wymer L, 2013, Methods for primary concentration of viruses from water samples: a review and meta-analysis of recent studies, Journal of Applied Microbiology, 115, 1-11.
- Chapron C D, Ballester N A, Fontaine J H, Frades C N, Margolin A B, 2000, Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated

cell culture-nested PCR procedure, Applied and Environmental Microbiology, 66:2520–2525.

- Chigor V N, Okoh A I, 2012, Quantitative RT-PCR Detection of Hepatitis A Virus, Rotaviruses and Enteroviruses in the Buffalo River and Source Water Dams in the Eastern Cape Province of South Africa, International Journal of Environmental Research in Public Health, 9, 4017-4032.
- Chinivasagam H N, Blackall P J, 2005, Investigation and application of methods for enumerating heterotrophs and Escherichia coli in the air within piggery sheds, Journal of Applied Microbiology, 98, 1137–1145.
- Chow J C, Watson J G, Green M C, Lowenthal D H, Bates B, Oslund W, Torres G, 2000, Cross-border transport and spatial variability of suspended particles in Mexicali and California's Imperial Valley, Atmospheric Environment, 34 (11) 1833–1843.
- Chuan S, Ellender R D, Johnson R A, Howell F G, 1983, Elution of Viruses from Coastal Sediments, Applied and Environmental Microbiology, 46(4): 797-804.
- Clark I N, Estes M K, Green K Y, Hansman G S, Knowles N J, Koopmans M K, Matson D O, Meyers G, Neill J D, Radford A, Smith A W, Studdert M J, Thiel H J, Vinjé J, 2012, Family Caliciviridae. In AMQ King, MJ Adams, EB Carstens, Lefkowitz, Virus taxonomy: Ninth REPORT of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, London, p. 977-986.
- Corrêa A A, Carratala A, Barardi C R M, Calvo M, Girones R, Bofill-Mas S, 2012, Comparative Inactivation of Murine Norovirus, Human Adenovirus, and Human JC Polyomavirus by Chlorine in Seawater, Applied and Environmental Microbiology, 78(18).
- Costantini V, Loisy F, Joens L, Le Guyader F S, Saif L J, 2006, Human and Animal Enteric Caliciviruses in Oysters from Different Coastal Regions of the United States, Applied and Environmental Microbiology, 72(3), 1800–1809.
- Cox C S, Wathes C M, 1995, Bioaerosols handbook, Lewis, Boca Raton.
- Croci L, De Medici D, Scalfaro C, Fiore A, Divizia M, Donia D, Cosentino A M, Moretti P, Costantini G, 2000, Determination of enteroviruses, hepatitis A virus, bacteriophages and Escherichia coli in Adriatic Sea mussels, Journal of Applied Microbiology, 88, 293–298.
- Crow S A, Ahern D G, Cook W L, Bourquin A W, 1975, Densities of bacteria and fungi in coastal surface films as determined by a membrane-adsorption procedure, Limnology and Oceanography, 20, 644–646.

- Cunliffe M, Schäfer H, Harrison E, Cleave S, Upstill-Goddard R, Murrell J C, 2008, Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary, ISME Journal, 2, 776–789
- Cunliffe M, Upstill-Goddard R C, Murrell J C, 2011, Microbiology of aquatic surface microlayers, FEMS Microbiological Reviews, 1–14.
- Cutler T D, Wanga C, Hoff S J, Zimmerman J J, 2012, Effect of temperature and relative humidity on ultraviolet (UV254) inactivation of airborne porcine respiratory and reproductive syndrome virus, Veterinary Microbiology, 159: 47–52.
- Danovaro R, Corinaldesi C, Dell'Anno A, Fuhrman J. A, Middelburg J. J, Noble R. T, Suttle C. A, 2011, Marineviruses and global climate change, FEMS Microbiology Reviews, 35, 993–1034.
- Dee S A, Deen J, Jacobson L, Rossow K D, Mahlum C, Pijoan C, 2005, Laboratory model to evaluate the role of aerosols in the transport of porcine reproductive and respiratory syndrome virus, Veterinary Research, 16, 501–504.
- Després V R, Nowoisky J F, Klose M, Conrad R, Andreae M O, 2007, Characterization of primary biogenic aerosol particles in urban, rural, and high-alpine air by DNA sequence and restriction fragment analysis of ribosomal RNA genes, Biogeosciences, 4, 1127-1141.
- Després V R, Huffman J A, Burrows S M, Hoose C, Safatov A S, Buyak G, Fröhlich-Nowoisky J, Elbert W, Andreae M, Pöschil U, Jaenicke R, Tellus B, 2012, Primary biological aerosol particles in the atmosphere: a review, Chemical and Physical Meteorology, 64, 15598.
- Desselberger U, 2000, Review article: Emerging and Re-emerging Infectious Diseases, Journal of Infection, 40, 3–15.
- Divizia M, Gabrieli R, Donia D, 2004, Waterborne gastroenteritis outbreak in Albania, Water Science and Technology, 50(1) 57-61.
- Domingo E, 2010, Mechanisms of viral emergence, Vet. Res., 41:38.
- Donaldson A I, Ferris N P, Gloster J, 1982, Air sampling of pigs infected with foot-and-mouth disease virus: comparison of Litton and cyclone samplers, Research Veterinary Science, 33, 384–385.
- Donaldson A I, Gibson C F, Oliver R, Hamblin C, Kitching R P, 1987, Infection of cattle by airborne foot-and-mouth disease virus: minimal doses with O1 and SAT 2 strains, Research Veterinary Science, 43, 339–346.

- Dongsheng D, 2006, From the smallest virus to the biggest gene: marching towards gene therapy for duchenne muscular dystrophy, Discov. Med, 6, 103-106.
- Dueñas C, Fernandez M C, Carretero J, Liger E, Cañete S, 2004, Long-term variation ofthe concentrations of long-lived Rn descendants and cosmogenic ^{7}Be and determination ofthe MRT ofaerosols, Atmospheric Environment, 38, 1291–1301.
- Duizer E, Bijkerk P, Rockx B, Groot A, Twisk F and Koopmans M, 2004, Inactivation of Caliciviruses, Applied and Environmental Microbiology, 70(8).4538–4543.
- Eden J S, Tanaka M M, Boni M F, Rawlinson W D, White P A, 2013, Recombination within the Pandemic Norovirus GII.4 Lineage, Journal of Virology, 87 (11): 6270–6282.
- Eischeid A C, Meyer J N, Linden K G, 2009, UV Disinfection of Adenoviruses: Molecular Indications of DNA Damage Efficiency, Applied and Environmental Microbiology, 75 (1) 23-28
- Elazhary M A, Derbyshire J B, 1979, Aerosol stability of bovine parainfluenza type 3 virus, Can. J. Comp. Med, 43:295–304.
- Enriquez C E, Hurst C J, Gerba C P, 1995, Survival of the enteric adenoviruses 40 and 41 in tap, sea, and waste water, Water Research, 29 (11) 2548–2553.
- Fannin K F, Vana S C, Jakubowski W, 1985, Effect of an Activated Sludge Wastewater Treatment Plant on Ambient Air Densities of Aerosols Containing Bacteria and Viruses, Applied and Environmental Microbiology, 49 (5) 1191-1196.
- Fierer N, Liu Z Z, Rodriguez-Hernandez M, Knight R, Henn M, 2008, Short-term temporal variability in airborne bacterial and fungal populations, Applied and Environmental Microbiology, 74, 200-207.
- Fong T-T, Griffin D W, Lipp E K, 2005, Molecular Assays for Targeting Human and Bovine Enteric Viruses in Coastal Waters and Their Application for Library-Independent Source Tracking, Applied and Environmental Microbiology, 71, 2070–2078.
- Fong T-T, Lipp E K, 2005, Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools, Microbiology and Molecular Biology reviews, 69 (2) 357-371.
- Fout G S, Martinson B C, Moyer M W N, Dahling D R, 2003, A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater, Applied and Environmental Microbioligy, 69 (6), 3158–3164.
- Fröhlich-Nowoisky J, Pickersgill D A, Després V R, Pöschl U, 2009, High diversity of fungi in air particulate matter, Proc. Natl. Acad. Sci. USA, 106, 12814-12819.

- Fumian T M, Leite J P G, Castello A A A, Caillou M S L, Miagostovich M P, 2010, Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration, Journal of Virological Methods, 170, 42–46.
- Fuzzi S, Andreae M O, Huebert B J, Kulmala M, Bond T C, 2006, Critical assessment of the current state of scientific knowledge, terminology, and research needs concerning the role organic aerosols in the atmosphere, climate, and global change, Atmospheric Chemistry and Physics, 6, 2017-2038.
- Garrett W D, 1965, Collection of slick-forming materials from the sea surface, Limnology and Oceanography, 10, 602–605.
- Georgakopoulos D G, Després V, Fröhlich-Nowoisky J, Psenner R, Ariya P A, 2009, Microbiology and atmospheric processes: biological, physical and chemical characterization of aerosol particles, Biogeosciences, 6, 721-737.
- Gerba C P, Gramos D M, Nwachuku N, 2002, Comparative Inactivation of Enteroviruses and Adenovirus 2 by UV Light, Applied and Environmental Microbiology, 68 (10) 5167–5169.
- Goyal S M, Adams W N, O'Malley M L, Lear D W, 1984, Human Pathogenic Viruses at Sewage Sludge Disposal Sites in the Middle Atlantic Region, Applied and Environmental Microbiology, 48 (4), 758-763.
- Grabow W, 1996, Waterborne diseases: Update on water quality assessment and control, Water SA, 22(2), 193-202.
- Grammatika M, Zimmerman W B, 2001, Microhydrodynamics offloatation process in the sea surface layer, Dynamics of Atmospheres and Ocean, 34, 327–348.
- Grant S H, 2010, Caliciviruses: Molecular and Cellular Virology, Scientific Press.
- Grassi T, Bagordo F, Ídolo A, Lugoli F, 2009, Rotavirus detection in environmental water samples by tangential flow ultrafiltration and RT-nested PCR, Environmental Monit Assessment.
- Green D H, Lewis G D, 1999, Comparative detection of enteric viruses in wastewaters, sediments and oysters by reverse transcription PCR and cell culture, Water Research, 33, 1195–1200.
- Griffin D W, Gibson C J, Lipp E K, Riley K, Paul J H, Rose J B, 1999, Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys, Applied and Environmental Microbiology, 65, 4118–4125.

- Griffin D W, Gibson III C J, Lipp E K, Riley K, Paul III J H, Rose J B, 2003, Detection of Viral Pathogens by Reverse Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys, Applied and Environmental Microbiology, 65(9): 4118-4125.
- Grohmann G S, 1997, Viruses, food and environment. Foodborne microorganisms of public health significance. North Sydney, Australia: Australian Institute of Food Science and Technology.
- Guillois-Bécel Y, Couturier E, Le Saux J C, Roque-Afonso A M, Le Guyader F S, Le Goas A, Pernès J, Le Bechec S, Briand A, Robert C, Dussaix E, Pommepuy M, Vaillant V, 2009, An oyster-associated hepatitis A outbreak in France in 2007 Euro Surveillance, 14(10).
- Guo H, Chu X, Hu J, 2010, Effect of Host Cells on Low- and Medium-Pressure UV Inactivation of Adenoviruses, Applied and Environmental Microbiology, 76 (21) 7068–7075.
- Gustafsson M E R, Franzén L G, 2000, Inland transport of marine aerosols in southern Sweden, Atmospheric Environment, 34, 313-325.
- Guttman-Bass N, Armon R, 1983, Concentration of Simian Rotavirus SA-11 from tap water by membrane filtration and organic flocculation, Applied and Environmental Microbiology, 45(3), 850– 855.
- Halliday M L, Kang L Y, Zhou T K, Hu M D, Pan Q C, Fu T Y, Huang Y S, Hu S L, 1991, An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China, Journl of Infectious Disease, 164, 852–859.
- Hamza I A, Jurzik L, Stang A, Sure K, Uberla K, Wilhelm M, 2009, Detection of human viruses in rivers of a densely-populated area in Germany using a virus adsorption method optimized for PCR analyses, Water Research, 43, 2657–2668.
- Hardy J T, 1982, The sea-surface microlayer – biology, chemistry and anthropogenic enrichment, Prog Oceanography, 11, 307–328.
- Harrach B, Benkő M, Both G W, Brown M, Davison A J, Echavarría M, Hess M, Jones M S, Kajon A, Lehmkuhl H D, Mautner V, Mittal S K, Wadell G., 2011, Family Adenoviridae. In: King A.M.Q., Adams M.J., Carstens E.B., Lefkowitz, E.J. (Eds.): Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego pp. 125–141.
- Harstad J B, 1965, Sampling submicron T1 bacteriophage aerosols, Applied and Environmental Microbiology, 13, 899–908.

- Harvey G W, 1965, Microlayer collection from the sea surface. A new method and initial results, Limnology and Oceanography, 11, 608–613.
- Harvey G W, Burzell L A, 1972, A simple microlayer method for small samples. Limnology and Oceanogrphy, 11, 156–157.
- Hasing M E, Hazes B, Lee B E, Preiksaitis J K, Pang X L, 2014, Detection and analysis of recombination in GII.4 norovirus strains causing gastroenteritis outbreaks in Alberta, Infection, Genetics and Evolution, 27: 181-192.
- Hermann J R, Hoff S J, Yoon K J, Burkhardt A C, Evans R B, Zimmerman J J, 2006, Optimization of a sampling system for recovery and detection of airborne porcine reproductive and respiratory syndrome virus and swine influenza virus, Applied and Environmental Microbiology, 72, 4811–4818.
- Hinds W C, 1999, Aerosol Technology: Properties. Behavior, and Measurement of airborne Particles 2nd.
- Hsu B-M, Chen C-H, Kung C-M, Wan M-T, Shen S-M, 2007, Evaluation of enterovirus recovery in surface water by different adsorption and elution procedures, Chemosphere, 66, 964–969.
- Hu X, Geng S, Wang X, Hu C, 2011, Inactivation and Photorepair of Enteric Pathogenic Microorganisms with Ultraviolet Irradiation, Environmental Engineering Science, 29 (6) 549 – 553.
- Huang Z, Deng X, Li Y, Su H, Li K, Guo Z, Zheng P, Xu H, He J, Zhang Q, Weng S, 2012, Structural insights into the classification of Mud Crab Reovirus, Virus Research, 166(1–2):116–120.
- Hundesa A, Maluquer C, Bofill-Mas S, Gimenez NA, Girones R, 2006, Identification of Human and Animal Adenoviruses and Polyomaviruses for Determination of Sources of Fecal Contamination in the Environment, Applied and Environmental Microbiology ,72(12) 7886-7893.
- Ijaz M K, Karim Y G, Sattar S A, Johnson-Lussenburg C M, 1987, Development of methods to study the survival of airborne viruses, Journal of Virological Methods, 18, 87-106.
- Jaenicke R, 2005, Abundance of cellular material and proteins in the atmosphere, Science, 308, 73.
- Jensen P A, Todd W F, Davis G N, Scarpino P V, 1992, Evaluation of eight bioaerosol samplers challenged with aerosols of free bacteria, Am Ind. Hyg. Assoc. Journal, 53:660–667.

- Jiang S, Noble R, Chu W, 2001, Human Adenoviruses and Coliphages in Urban Runoff Impacted Coastal Waters of Southern California, Applied and Environmentl Microbiology, 67 (1) 179 – 184.
- Jiang Y J, Liao G Y, Zhao W, Sun M B, Qian Y, Bian C X, Jiang S D, 2004, Detection of infectious hepatitis A virus by integrated cell culture/strand-specific reverse transcriptase-polymerase chain reaction, Journal of Applied Microbiology, 97, 1105–1112.
- Joseph H M, Ballmann M Z, Garner M M, Hanley C S, Berlinski R, Erdélyi K, Childress A L, Fish S S, Harrach B, Wellehan J F X, 2014, A novel siadenovirus detected in the kidneys and liver of Gouldian finches (Erythura gouldiae), Veterinary Microbiology, doi.org/10.1016/j.vetmic.2014.04.006
- Jothikumar N, Cromeans T L, Hill V R, Lu X, Sobsey M D, Erdman D D, 2005, Quantitative Real-Time PCR Assays for Detection of Human Adenoviruses and Identification of Serotypes 40 and 41, Applied and Environmental Microbiology, 71 (6) 3131–3136.
- Juozaitis A, Willeke K, Grinshpun S A, Donnelly J, 1994, Impaction onto a glass slide or agar versus impingement into a liquid for the collection and recovery of airborne microorganisms, Applied and Environmental Microbiology, 60, 861–870.
- Karim Y G, Ijaz M K, Sattar S A, Johnson-Lussenburg C M, 1985, Effect of relative humidity on the airborne survival of rhinovirus-14, Can. Journal of Microbiology, 31, 1058–1061.
- Katamaya H, Shimasaki A, Ohgaki S, 2002, Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater, Applied and Environmental Microbiology, 68(3), 1033–1039.
- Katayama K, Miyoshi T, Uchino K, 2004, Novel Recombinant Sapovirus. Emerging Infectious Diseases, 10(10).
- Katzenelson E, Fattal B, Hostovesky T, 1976, Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water, Applied and Environmental Microbiology, 32(4), 638–639.
- Kingsley D H, Meade G K, Richards G P, 2002, Detection of both Hepatitis A Virus and Norwalk-Like Virus in Imported Clams Associated with Food-Borne Illness, Applied and Environmental Microbiology, 68(8), 3914–3918.
- Kingsley D H, 2013, High Pressure Processing and its Application to the Challenge of Virus-Contaminated Foods, Food Environmental Virology, 5, 1–12.

- Kjelleberg S, Stenstrom T A, Odham G, 1979, Comparative study of different hydrophobic devices for sampling liquid surface films and adherent microorganisms, Marithim Biology, 53, 21–25.
- Klassen R D, Roberge P R, 1999, Aerosol transport modeling as an aid to understanding atmospheric corrosivity patterns, Materials & Design, 20 (4), 159–168.
- Ko G, Cromeans T L, Sobsey M D, 2003, Detection of Infectious Adenovirus in Cell Culture by mRNA Reverse Transcription-PCR, Applied and Environmental Microbiology, 69(12), 7377-7384.
- Koivunen J, Heinonen-Tanski H, 2005, Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments, Water Research, 39, 1519.
- Kovac K, Gutierrez-Aguirre I, Banjac M, Peterka M, Prijatelj M P, Ravnikar M, Mijovski J Z, Schultze A C, Raspor P, 2009, A novel method for concentrating hepatitis A virus and caliciviruses from bottled water, Journal of Virological Methods, 162, 272–275.
- Kuznetsova M, Lee C, Aller J, 2005, Characterization of the proteinaceous matter in marine aerosols, Marine Chemistry, 96, 359– 377.
- La Rosa G, Fratini M, Libera S, Iaconelli M, Muscillo M, 2012, Emerging and potentially emerging viruses in water environments, Ann Ist Super Sanità, Vol. 48 (4) 397-406.
- Lawton J A, Estes M K, Prasad B V V, 2000, Mechanism of genome transcription in segmented dsRNA viruses. Advances in Virus Research, 55: 185–214, IN1–IN8, 215–229.
- Lebecka M G, McCarthya T A, Ana W, 2009, Emergent US adenovirus 3 strains associated with an epidemic and serious disease, Journal of Clinical Virology, 46(4), 331-336.
- Lee S H, Kim S J, 2002, Detection of infectious enteroviruses and Adenoviruses in tap water in urban areas in Korea, Water Research, 36(1), 248–256.
- Lee S H, Lee C, Lee K W, Cho H B, Kim S J, 2005, The simultaneous detection of both enteroviruses and adenoviruses in environmental water samples including tap water with an integrated cell culture-multiplex nested PCR procedure, Journal of Applied Microbiology, 98, 1020–1029.

- Lee J I, Lee G-C, Chung J Y, Han T H, Lee Y K, Kim M S, Lee C H, 2012, Detection and molecular characterization of adenoviruses in Korean children hospitalized with acute gastroenteritis, Microbiology and Immunology, 56 (8), 523–528.
- Le-Guyader F L, Apaire-Marchais V, Brillet J, Billaudel S, 1983, Use of Genomic Probes To Detect Hepatitis A Virus and Enterovirus RNAs in Wild Shellfish and Relationship of Viral Contamination to Bacterial Contamination, Applied and Environmental Microbiology, 59(11), 3963-3968.
- Le-Guyader F S, Parnaudeau S, Schaeffer J, 2009, Detection and Quantification of Noroviruses in Shellfish, Applied and Environmental Microbiology, 75(3), 618-624.
- Leshem E, Wikswo M, Barclay L, Brandt E, Storm W, Salehi E, DeSalvo T, Davis T, Saupe A, Dobbins G, Booth H A, Biggs C, Garman K, Woron A M, Parashar U D, Vinjé J, Hall A J, 2013, Effects and Clinical Significance of GII.4 Sydney Norovirus, United States, 2012–2013, Emerging Infectious Diseases, 19 (8): 1231-1238.
- Leveque N, Laurent A, 2008, A Novel Mode of Transmission for Human Enterovirus Infection Is Swimming in Contaminated Seawater: Implications in Public Health and in Epidemiological Surveillance, Clinical Infectious, 47, 624–626.
- Li X, Liu Q, Bi X, Sheng G, Fu J, Ran P, Li B, 2008, An in vitro model to evaluate virus aerosol characteristics using a GFP-expressing adenovirus, Journal of Medical Microbiology, 57, 1335–1339.
- Lim E S, Caoa S, Holtzb L R, Antonioc M, Stined O C, Wanga D, 2014, Discovery of rosavirus 2, a novel variant of a rodent-associated picornavirus, in children from The Gambia, Virology, 454-455: 25-33.
- Lin X, Willeke K, Ulevicius V, Grinshpun S A, 1997, Effect ofsampling time on the collection efficiency of the all-glass impingers, Am. Ind. Hyg. Assoc. J, 58, 480–488.
- Lin W H, Li C S, 1998, The effect of sampling time and flow rates on the bioefficiency of three fungal spore sampling methods, Aerosol Science Technology, 28, 511–522.
- Lin W H, Li C S, 1999, Collection efficiency and culturability of impingement into a liquid for bioaerosols of fungal spores and yeast cells, Aerosol Science Technology, 30, 109–118.
- Lin X, Reponen T, Willeke K, Wang Z, Grinshpun S, Trunov M, 2000, Survival of airborne microorganisms during swirling aerosol collection, Aerosol Science Technology, 32, 184–196.

- Linden K G, Thurston J, Schaefer R, Malley J P, 2007, Enhanced UV Inactivation of Adenoviruses under Polychromatic UV Lamps, Applied and Environmental Microbiology, 73(23), 7571-7574.
- Lipp E K, Lukasik J, Rose J B, 2001, Human enteric viruses and parasites in the marine environment, Methods Microbiology, 30, 559–588.
- Lipp E K, Jarrell J L., Griffin D W, Lukasik J, Rose B J, 2002, Preliminary evidence for human fecal contamination in corals of the Florida Keys, USA. Maritim Pollution Bulletin, 44, 666–670.
- Liu W; Wu S; Xiong Y; Li T; Wen Z; Yan M; Qin K; Liu Y; Wu J, 2014, Co-Circulation and Genomic Recombination of Coxsackievirus A16 and Enterovirus 71 during a Large Outbreak of Hand, Foot, and Mouth Disease in Central China, DOI: 10.1371.
- Lo S, Gilbert J, Hetrick F, 1976, Stability of Human Enteroviruses in Estuarine and Marine Waters, Applied and Environmental Microbiology, 32 (2) 245-249.
- Loisy F, Atmar R L, Cohen J, Bosch A, Le Guyader F S, 2000, Rotavirus VLP2/6: a new tool for tracking rotavirus in the marine environment, Research in Microbiology, 155 (7) 575–578.
- Lugoli F, Leopizzi M I, De Donno A, 2009, Viral Indicators of Hygienic-Sanitary Quality: Detection of Somatic Coliphages in the Southern Adriatic Sea, Water Qual Expo Health, 1, 171-177.
- Lukasik J, Scott T M, Andryshak D, Farrah S R, 2000, Influence of salts on virus adsorption to microporous filters, Applied and Environmental Microbiology, 66, 2914–2920.
- Mahony J B, 2008, Detection of Respiratory Viruses by Molecular Methods, Clinical Microbiology Reviews, 21(4), 716-747.
- Mahy B W J, Van Regenmortel M H V, 2009, Desk Encyclopedia of General Virology, Elsevier.Oxford.
- Maron P - A, Lejon D P H, Carvalho E, Bizet K, Lemanceau P, 2005, Assessing genetic structure and diversity of airborne bacterial communities by DNA Finger-printing and 16S rDNA clone library, Atmospherical Environment, 39, 3687-3695.
- Marks R, Kruczalak K, Jankowska K, Michalska M, 2001, Bacteria and fungi in air over the Gulf of Gdańsk and Baltic sea, Journal of Aerosol Science, 32, 237-250.
- Matthias-Maser S, Jaenicke R, 1995, The size distribution of primary biological aerosol particles with radii > 0.2 μm in an urban/rural influenced region, Atmospheric Research, 39 (4) 279–286.

- Melnick J.L, Gerba C.P, Wallis C, 1978, Viruses in water, Bulletin of the World Health Organization, 56 (4): 499-508.
- Mesquita J R, Vaz L, Cerqueira S, Castilho F, Santos R, Manso C F, Romalde J L, Nascimento M S J, Monteiro S, 2011, Norovirus, hepatitis A virus and enterovirus presence in shell!sh from high quality harvesting areas in Portugal, Food Microbiology, 1-6.
- Michen, B. and Graule, T., 2010, Isoelectric points of viruses, Journal of Applied Microbiology, 109, 388–397.
- Mikalsen A B, Nilsen P, FrøystadSaugen M, Lindmo K, Eliassen T M, Rode M, Evensen Ø, 2014, Characterization of a Novel Calicivirus Causing Systemic Infection in Atlantic Salmon (Salmo salar L.): Proposal for a New Genus of Caliciviridae, PLOS ONE, DOI: 10.1371/journal.pone.0107132.
- Ming H X, Fan J F, Wu L J, Liang Y B, 2013, Prevalence of Human Enteric Viruses and a Potential Indicator of Contamination in Shellfish in China, Journal of Food Safety, 33 (2), 209–214.
- Moorthy K K, Satheesh S K, Murthy B V K, 1997, Investigations of marine aerosols over tropical Indian Ocean, Journal of Geophysical Research, 102, (18), 827 –842.
- Moresco V, Viancelli A, Nascimento M A, Souza D S M, Ramos A P D, Garcia L A T, Simões C M O, Barardi C R M, 2012, Microbiological and physicochemical analysis of the coastal waters of southern Brazil, Marine Pollution Bulletin, 64 (1) 40-48.
- Morris C E, Sands D C, Bardin M, Jaenicke R, Vogel B, Leyronas C, Ariya P A, Psenner R, 2011, Microbiology and atmospheric processes: research challenges concerning the impact of airborne micro-organisms on the atmosphere and climate, Biogeosciences, 8, 17–25.
- Mubareka S, Lowen A C, Steel J, Coates A L, García-Sastre A, Palese P, 2009, Transmission of Influenza Virus via Aerosols and Fomites in the Guinea Pig Model, The Journal of Infectious Diseases; 199,858 –65.
- Muscillo M, Pourshaban M, Iaconelli M, Fontana S, Di Grazia A, Manzara S, Fadda G, Santangelo R, La Rosa G, 2008, Detection and quantification of human Adenoviruses in surface waters by nested PCR, TaqMan Real-Time PCR and cell culture assays, Water, Air, Soil Pollution, 191, 83–93.
- Myrmel M, Berg E M M, Grinde B, Rimstad E, 2006, Enteric viruses in inlet and outlet samples from sewage treatment plants, Journal of Water Health, 197–209.

- Nenonen N P, Hannoun C, Horal P, Hernroth B, Bergström T, 2008, Tracing of Norovirus Outbreak Strains in Mussels Collected near Sewage Effluents, Applied and Environmental Microbiology, 74 (8), 2544–2549.
- Nicholson K W, 1995, Physical aspects if bioaerosol sampling and deposition. In: Bioaerosols Handbook (eds Cox, C. S. and C. M. Wathes), CRC-Press, Boca Raton, Florida, USA, 27-47.
- Nishida T, Kimura H, Saitoh M, 2003, Detection, Quantitation, and Phylogenetic Analysis of Noroviruses in Japanese Oysters, Applied and Environmental Microbiology, 69 (10), 5782-5786.
- Noble R T, Fuhrman J A, 1997, Virus Decay and Its Causes in Coastal Waters, Applied and Environmental Microbiology, 63(1),77-83.
- Noble R T, Fuhrman J A, 1998, Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria, Aquatic Microbial Ecology, 14, 113–118.
- Noble R T, Allen S M, Blackwood A D, Chu W, Jiang S C, Lovelace G L, Sobsey M D, Stewart J R, Wait D A, 2003, Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial course tracking comparison study, Journal of Water Health, 1, 195–207.
- O'Dowd C D, Facchini M C, Cavalli F, Ceburnis D, Mircea M, Decesari S, Fuzzi S, Yoon Y J, Putaud J-P, 2004, Biogenicall driven organic contribution to marine aerosol, Nature, 431, 676–680.
- Parada V S, Sintes E, Weinbauer M, Herndl G, 2005, Dynamics in the Sea Surface Microlayer in an anticyclonic eddy of the Northwestern Mediterranean Sea, Journal of Virioplankton.
- Paul J H, Rose J B, Jiang S C, Kellogg C A, Dickson L, 1993, Distribution of Viral Abundance in the Reef Environment of Key Largo, Florida, Applied and Environmental Microbiology 59 (3), 718-724.
- Parsons T R, Maita Y, Lalli C M, 1989, A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford, pp. 172.
- Peccia J, Hernandez M, 2006, Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review, Atmospheric Environment, 40, 3941–3961.
- Percival S, Chalmers R, Embrey M, Hunter P, Sellwod J, Wyn-Jones P, 2004, Microbiology of Waterborne Diseases, Viruses, Elsevier Academic Press, London, 1st edn, pp. 337–452.

- Peruski A H, Peruski Jr. L F, 2004, Immunological Methods for Detection and Identification of Infectious Disease and Biological Warfare Agents, Clinical and Diagnostic Laboratory Immunology, 10 (4), 506–513.
- Pianetti A, Baffone W, Citterio B, Caçarola A, Bruscoline F, Salvaggio L, 2000, Presence of enteroviruses and reoviruses in the waters of the Italian coast of the Adriatic Sea, Epidemiology and Infections, 125, 455-462.
- Piao J, Jiang J, Xu B, Wang X, Guan Y, Wu W, Zhang Y, Huang X, Wang P, Zhao J, Kang X,Cao Y, Zheng Y, Jiang Y, Li Y, Yang Y, Liu L, Hua J, Weijun C, 2012, Simultaneous Detection and Identification of Enteric Viruses by PCR-Mass Assay, Plos One, 7 (8).
- Pina S, Puig M, Lucena F, Jofre J, Girones R, 1998, Viral Pollution in the Environment and in Shellfish: Human Adenovirus Detection by PCR as an Index of Human Viruses, Applied and Environmental Microbiology, 64 (9), 3376-3382.
- Pintó R M, Alegre D, Dominguez A, El Senousy W M, Sanchez G, Villena C, Costafreda M I, Aragones L, Bosch A, 2007, Hepatitis A virus in urban sewage from two Mediterranean countries, Epidemiology and Infections, 135, 270–273.
- Polaczyck A L, Roberts J M, Hill V R, 2007, Evaluation of 1MDS electropositive microfilters to simultaneous recovery of multiple microbe classes from tap water, Journal of Microbiological Methods, 68, 260–266.
- Polz M F, Cavanaugh C M, 1998, Bias in template-toproduct ratios in multi template PCR, Applied and Environmental Microbiology, 64, 3724-3730.
- Pommepuy M, Le Guyader F, 1998, Molecular approaches to measuring microbial marine pollution, Current Opinion in Biotechnology, 9, 292–299.
- Pond K, 2005, Water recreation and disease: plausibility of associated infections: Acute Effects, Sequelae and Mortality, World Health Organization.
- Pöschl U, 2005, Climate and Health Effects. Atmospheric Aerosols: Composition, Transformation, Climate and Health Effects, Atmospheric Chemistry, 44, 7520 – 7540.
- Pósfai M, Simonics R, Li J, Hobbs P V, Buseck P R, 2003, Individual aerosol particles from biomass burning in southern Africa: Compositions and size distributions of carbonaceous particles, Journal of GeophYsical Research, 108 (19).
- Prata C, Ribeiro A, Cunha A, Gomes N C M, Almeida A, 2012, Ultracentrifugation as a direct method to concentrate viruses in environmental waters: virus-like particles enumeration as a new approach to determine the efficiency of recovery, Journal of Microbiological Methods, 14 (1), 64-70.

- Puig M, Jofre J, Lucena F, Allard A, Wadell G, Girones R, 1994, Detection of Adenoviruses and enteroviruses in polluted water by nested PCR amplification, Applied and Environmental Microbiology, 60(8), 2963–2970.
- Pusch D, Oh D Y, Wolf S, Dumke R, Schröter-Bobsin U, Höhme M, Röske I, Schreier E, 2005, Detection of enteric viruses and bacterial indicators in German environmental waters, Archives of Virology, 150, 929-947.
- Quek P H, Hu J Y, 2008, Indicators for photoreactivation and dark repair studies for following ultraviolet disinfection, Journal of Industrial Microbiology and Biotechnology, 35, 533.
- Rao V C, Seidel K N, Goyal S M, Metcalf T C, Melnick J L, 1984, Isolation of enteroviruses from water, suspended solids and sediments from Galveston bay; survival of poliovirus and rotavirus adsorbed to sediments, Applied and Environmental Microbiology, 48 (2), 404–409.
- Reynolds K A, Gerba C P, Pepper I L, 1996, Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. Applied and Environmental Microbiology, 62, 1424–1427.
- Reynolds K A, Gerba C P, Abbaszadegan M, Pepper L L, 2001, ICC/PCR detection of enteroviruses and hepatitis A virus in environmental samples, Can. Journal of Microbiology, 47, 153–157.
- Reynolds K A, 2004, Integrated cell culture/PCR for detection of enteric viruses in environmental samples, Methods of Molecular Biology, 268:69–78.
- Rigotto C, Hanley K, Rochelle P A, De Leon R, Barardi C R M, Yates M V, 2011, Survival of Adenovirus Types 2 and 41 in Surface and Ground Waters Measured by a Plaque Assay, Environmental Science Technology, 45 (9), 4145–4150.
- Robinson C M., Singh G, Henquell C, Wals M P, Peigue-Lafeuille H, Seto D, Jones M S, Dyer D W, Chodosh J, 2011, Computational analysis and identification of an emergent human adenovirus pathogen implicated in a respiratory fatality, Virology, 409, 141–147.
- Robinson C M, Setob D, JonescM S, Dyerd D W, Chodosh J, 2011, Molecular evolution of human species D adenoviruses, Infection, Genetics and Evolution, 11(6), 1208–1217.
- Rodriguez-Diaz J, Querales L, Caraballo L, Vizzi E, Liprandi F, Takiff H, Betancourt W Q, 2009, Detection and characterization of waterborne gastroenteritis viruses in urban sewage and sewagepolluted river waters in Caracas, Venezuela, Applied and Environmental Microbiology, 75(2), 387–394.

- Romalde J L, Torrado I, Ribao C, Barja J. L, 2001, Global market: shellfish imports as a source of reemerging food-borne hepatitis A virus infections in Spain, Int Microbioogy, 4, 223–226.
- Romalde J L, Área E, Sánchez G, Ribao C, Torrado I, Abad X, Pinto R. M, Barja J. L and Bosch A, 2002, Prevalence of enterovirus and hepatitis A virus in bivalve molluscs from Galicia (NW Spain): inadequacy of the EU standards of microbiological quality, International Journal of Food Microbiology, 74,119–130.
- Rosa G, Fontana S, Di Grazia A, 2007, Molecular Identification and Genetic Analysis of Norovirus Genogroups I and II in Water Environments: Comparative Analysis of Different Reverse Transcription-PCR Assays, Applied and Environmental Microbiology, 73(13), 4152–4161.
- Russell P B, Bergstrom R W, Shinozuka Y, Clarke A D, DeCarlo P F, Jimenez J L, Livingston J M, Redemann J, Dubovik O, Strawa A, 2010, Absorption Angstrom Exponent in AERONET and related data as an indicator of aerosol composition, Atmospherical Chemistry Phys., 10, 1155–1169.
- Rzeżutka A, Cook N, 2004, Survival of human enteric viruses in the environment and food, FEMS Microbiology Reviews, 28, 441–453.
- Sagripanti J-L, Lytle D C, 2007, Inactivation of Influenza Virus by Solar Radiation, Photochemistry and Photobiology, 83, 1278–1282.
- Schoenbaum M A, Zimmerman J J, Beran G W, Murphy D P,1990, Survival of pseudorabies virus in aerosol, American Journal of Veterinary Research, 51, 331–333.
- Schwab K J, De Leon R, Sobsey M, 1995, Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A viruses, and norwalk viruses by reverse transcription-PCR, Applied and Environmental Microbiology, 61 (2) 527–531.
- Sedmak G, Bina D, Macdonald J, Couillard L, 2005, Nine-year study of the occurrence of culturable viruses in source water for two drinking water treatment plants and the influent and effluent of a wastewater-treatment plant in Milwakee, Wisconsin, Applied and Environmental Microbiology, 71, 1042–1050.
- Seo K; Lee J E; Lim M Y; Ko G, 2012, Effect of Temperature, pH, and NaCl on the Inactivation Kinetics of Murine Norovirus, Journal of Food Protection, 75, (3), 533-540.

- Shields P A, Farrah S R, 1986, Concentration of viruses in beef extract by flocculation with ammonium sulfate, Applied and Environmental Microbiology, 51(1) 211–213.
- Smith A W, Skilling D E, Cherry N, 1993, Calicivirus Emergence from Ocean Reservoirs: Zoonotic and Interspecies Movements, Emerging Infectious Diseases 4(1).
- Sommer R, Pribil W, Appelt S, Gehringer P, Eschweiler H, Leth H, Cabaj A, Haider T, 2001, Inactivation of bacteriophages in water by means of non-ionizing (UV253.7nm) and ionizing (gamma) radiation: A comparative approach, Water Research, 35, 3109.
- Saint-Louis R, Pelletier E, 2004, Sea-to-air flux of contaminants via bubbles bursting, An experimental approach for tributyltin, Marine Chemistry, 84, 211 – 224.
- Stolze B, Kaaden O R, 1989, Efficient medium for impingement and storage of enveloped viruses, Zentbl. Vetmed, 36,161–167.
- Straub T M, Pepper I L, Gerba C P, 1995, Removal of PCR inhibiting substances in sewage sludge amended soil. Water Sci. Technol. 31:311–315.
- Sun J, Ariya P A, 2006, Atmospheric organic and bioaerosols as cloud condensation nuclei: A review, Atmospherical Environment, 40, 795–820.
- Suttle C A, Chen F, 1992, Mechanisms and Rates of Decay of Marine Viruses in Seawater, Applied and Environmental Microbiology, 58(11) 3721-3729.
- Suttle C A, 2005, Viruses in the sea, Nature, 437, 356-361.
- Suttle C A, 2007, Marine viruses — major players in the global ecosystem, Nature, 5, 801-812.
- Symonds E M, Breitbart M, 2014, Affordable Enteric Virus Detection Techniques Are Needed to Support Changing Paradigms in Water Quality Management, Clean Soil Air Wate,. doi: 10.1002/clen.201400235
- Tabor E, 2007, Emerging Viruses in Human population, V.16, 1ª edition. Elsevier.
- Terzieva, S, Donnelly J, Ulevicius V, Grinshpun S A, Willeke K, Stelma G N, Brenner K P, 1996, Comparison of methods for detection and enumeration of airborne microorganisms collected by liquid impingement, Applied and Environmental Microbiology, 62, 2264–2272.
- Thurston-Enriquez J A, Haas C N, Jacangelo J, Riley K, Gerba C P, 2005, Inactivation of Feline Calicivirus and Adenovirus Type 40 by UV Radiation, Applied and Environmental Microbiology, 69 (1) 577-582.

- Tsai Y L, Sobsey M D, Sangermano L R, Palmer C J, 1993, Simple Method of Concentrating Enteroviruses and Hepatitis A Virus from Sewage and Ocean Water for Rapid Detection by Reverse Transcriptase-Polymerase Chain Reaction, Applied and Environmental Microbiology, 59 (10), 3488-3491.
- Vega E, Barclay L, Gregoricus N, Shirley S H, Lee D, Vinjé J, 2014, Genotypic and Epidemiologic Trends of Norovirus Outbreaks in the United States, 2009 to 2013, Journal of Clinical Microbiology, 52 (1): 147–155.
- Verrault D, Moineau S, Duchaine C, 2008, Methods for Sampling of Airborne Viruses, Microbiology and Molecular Biology Reviews, 72 (3) 413-444.
- Victoria J G., Kapoor A, Li L, Blinkova O, Slikas B, Wang C, Naeem A., Zaidi S, Delwart E, 2009, Metagenomic analysis of viruses in stool samples from children with acute flaccid paralisys, Journal of Virology, 83(9) 4542–4651.
- Vieira C B, Mendes A C O,Guimarães F R, Fumian T M, Leite J P G, Gaspar A M C, Miagostovich M P, 2012, Detection of enteric viruses in recreational waters of an urban lagoon in the city of Rio de Janeiro, Brazil, Mem Inst Oswaldo Cruz, 107(6) 778-784.
- Voevodin A F, Marx A P, 2009, Simian Virology, John Wiley and Sons.
- Weinbauer M G, Christaki U, Nedoma J, Karel S, 2004, Comparing the effects of resource enrichment and grazing on viral production in a meso-eutrophic reservoir, Aquatic Microbial Ecology, 31 (2) 137-144.
- Wetz J J, Lipp E K, Griffin D W, Lukasik J, Wait D, Sobsey M D, Scott T M, Rose J B, 2004, Presence, infectivity, and stability of enteric viruses in seawater: relationship to marine water quality in the Florida Keys, Marine Pollution Bulletin 48, 698–704.
- White D. O, Fenner F, 1994, Medical virology, Gulf Professional Publishing.
- Williams P M, Carlucci A F, Henrichs S M, Vanvleet E S, Horrigan S G, Reid F M H, Robertson K J, 1986, Chemical and microbiological studies of sea-surface films in the southern gulf of California and off the west-coast of Baja-California, Mar Chem 19, 17–98.
- Wilson I, 1997, Inhibition and facilitation of nucleic acid amplification, Applied and Environmental Microbiology 63, 3741–3751.
- Woods J W, 2013, Waterborne Diseases of the Ocean, Enteric Viruses. Infectious Diseases, 381-398.
- Wyn-Jones A P, Sellwood J, 2001, Enteric viruses in the aquatic environment. A Review, Journal of Applied Microbiology 91, 945-962.

- Xagoraraki I, Kuo D H-W, Wong K, Wong M, Rose J B, 2007, Occurrence of Human Adenoviruses at Two Recreational Beaches of the Great Lakes, Applied and Environmental Microbiology, 73 (24) 7874–7881.
- Yates M V, Gerba C P, Kelley L M, 1985, Virus persistence in groundwater. Applied and Environmental Microbiology, 49(4) 778-781
- Yeh H Y, Hwang Y C, Yates M V, 2008, Detection of Hepatitis A Virus by Using a Combined Cell Culture-Molecular Beacon Assay, Applied and Environmental Microbiology, 74 (7) 2239–2243.
- Young C, Burghoff R, Keim L, Minak-Bernero V, Lute J, Hinton S, 1993, Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils, Applied and Environmental Microbiology, 59,1972–1974.
- Zhao G, Krishnamurthy S, Cai Z, Popov V L, Rosa A P T, Guzman H, Cao S, Virgin H W, Tesh R B, Wang D, 2013, Identification of Novel Viruses Using VirusHunter -- an Automated Data Analysis Pipeline, PLOS ONE, DOI: 10.1371/journal.pone.007847.

Chapter 2 - Ultracentrifugation as a direct method to concentrate virus in environmental waters: virus-like particle enumeration as a new approach to determine the efficiency of recovery

Ultracentrifugation as a direct method to concentrate virus in environmental waters: virus-like particle enumeration as a new approach to determine the efficiency of recovery

Catarina Prata, Andreia Ribeiro, Ângela Cunha, Newton Gomes, Adelaide Almeida

Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

Abstract

Some health important enteric viruses are considered to be emerging waterborne pathogens and so the improvement of detection of these viruses in the aquatic environment is one of the most important steps in dealing with these pathogens. Since these viruses may be present in low numbers in water, it is necessary to concentrate water samples before viral detection. Although there are several methods to concentrate viruses in environmental waters, all present some drawbacks and consequently the method should be chosen that, despite its limitations, is adequate to achieve the aim of each study. As the effectiveness of the concentration methods is evaluated by determining the efficiency of viral recovery after concentration, it is important to use a simple and effective approach to evaluate their recovery efficiency. In this work ultracentrifugation, usually used as a secondary step for viral concentration, was evaluated as the main method to concentrate directly virus in environmental water samples, using the microscopic enumeration of virus-like particles (VLP) as a new approach to estimate the efficiency of recovery. As the flocculation method is currently employed to concentrate virus in environmental waters, it was also used in this study to assess the efficiency of the ultracentrifugation as the main viral concentration method in environmental waters. The results of this study indicate that ultracentrifugation is an adequate approach to concentrate virus directly from environmental waters (recovery percentages between 66 and 72% in wastewaters and between 66 and 76% in recreational waters) and that the determination of VLP by epifluorescence microscopy is a simple, fast and cheap alternative approach to determine the recovery efficiency of the viral concentration methods.

Introduction

Health relevant enteric viruses are nowadays considered to be emerging waterborne pathogens (Noble and Fuhrman, 1998; Sedmark et al, 2005; Albinana-Gimenez et al, 2006) increasing the concern over the discharge of human enteric viruses not only into fresh water but also into estuarine and marine environments. In these environments they represent a health hazard in areas that are used for recreational purpose or from which shellfish are harvested for human consumption. The presence of those viruses in the aquatic environment represents a large problem for human health, economy and environmental ecology (Lee and Kim, 2002, Hamza et al, 2009; Rodriguez-Diaz et al, 2009). A large number of human enteric viruses have been shown to be discharged into marine waters by offshore sewage outfalls and they have also been detected in coastal water polluted by sewage treatment plants and septic tanks (LaBelle et al, 1980). Individuals suffering from diarrhea or hepatitis release a large number of viruses, values greater than 10^{13} and 10^{10} viral particles per gram of stool, respectively (Caballero et al, 2003; Costafreda et al, 2006; Ozawa et al, 2007). More than 140 types of virus that cause a variety of diseases to humans, which include hepatitis, gastroenteritis, meningitis, fever, influenza, respiratory disease, conjunctivitis, among others, can be found in wastewaters (Bosch et al, 2008; Hamza et al, 2009; Puig et al, 1994) However, only a small number of viruses is epidemiologically relevant (Bosch, 1998) and the most relevant viral pathogens found in water are the norovirus, rotavirus, adenovirus, astrovirus, enterovirus and hepatovirus (Ozawa et al, 2007; Bosch et al, 2008). The basic steps in the virological analysis of environmental water are sampling, concentration, decontamination/removal of inhibitors and specific virus detection. Concentration is a critical step, since the viruses may be present in such low numbers that it is necessary to concentrate and reduce the volume of the sample to a few millilitres (Tsai et al, 1993; Bosch, 1998). The absence of viral concentration methods with high recuperation efficiency has been indicated as a primary reason for the low number of studies in the area of environmental virology. A variety of strategies have been used to concentrate virus from water samples, which are based mostly on adsorption–elution techniques, flocculation, ultrafiltration and ultracentrifugation. During the last decade, a new chromatographic medium, monolithic supports, was also developed and applied successfully to the concentration of several viruses (Branovic et al, 2003; Kramberger et al, 2004; Gutierez-Aguirre et al, 2008). However, there is not yet a single method that enables a highly efficient concentration of all viruses (Block and Schwartzbrod, 1989; Bosch et al, 2008; Albinana-Gimenez et al, 2009). A good concentration method should fulfil several criteria, it should be technically simple, be fast, provide high viral recovery, be adequate for a wide range of viruses,

provide a small volume of concentrate, be cost effective (Bosch, 1998) and should not interfere with viral community structure (Angly et al, 2006). The last aspect is very important, namely when concentrated samples are used to get a global view of viral community composition, using for instance a high throughput DNA/cDNA sequencing approach. Flocculation methods are most frequently used to concentrate virus in environmental waters because they allow us to process large volumes of water (Katzenelson et al, 1976; Puig et al, 1994; Calgua et al, 2008) relative to ultrafiltration methods (Muscillo et al, 2008; Kovac et al, 2009). However, for these methods the electrostatic interaction between the virus and the surface of the filters/flakes depends on various factors, including viral isoelectric points, water pH, and salt concentration (Hsu et al, 2007; Polaczyck et al, 2007; Victoria et al, 2009), which imply water sample manipulation. They are based on the ability of protein flocculation at acid pH, getting the virus trapped in protein flakes which are then released after dissolution of the flakes (Percival et al, 2004). However, not only viruses are concentrated but also PCR inhibitory substances (Bosch, 1998; Fong and Lipp, 2005). Ultrafiltration methods are an alternative to adsorption–elution and flocculation techniques and have been shown to be efficient to recover viruses from raw and treated sewage, surface waters and wastewaters (Fong and Lipp, 2005; Grassi et al, 2010). Ultracentrifugation is a good alternative method for viral concentration in environmental water samples since it requires minimal manipulation, samples can be processed under natural pH and an elution step is not needed. By ultracentrifugation it is possible to concentrate all viruses in a sample, by using a sufficient g-force during an adequate period of time (Ozawa et al, 2007). Moreover, the time needed to perform this technique is reduced, when compared with flocculation methods, and it does not introduce any PCR inhibitory substance and, consequently, concentrated samples can be successfully used for molecular detection. Although it is difficult to process large volumes of water with this method it is possible to reduce the volume of the sample to fewer millilitres than with adsorption–elution and flocculation methods. The comparison among different concentration methods is difficult because it is necessary to take into account many variables (e.g. type of sample, volume of water and the methods used to determinate the recovery efficiency). Nevertheless, in general, for environmental waters, the ultracentrifugation method allows virus recovery efficiencies similar to those of adsorption–elution and flocculation methods (Guttman-Bass and Armon, 1983; Shields and Farrah, 1986; Puig et al, 1994; Calgua et al, 2008). The efficiency of viral recovery after concentration is usually determined by plaque assay approaches or real time PCR (Puig et al, 1994; Calgua et al, 2008; Lambertini et al, 2008; Muscillo et al, 2008). A viral suspension of known concentration is added to the sample and, after concentration,

plaques of lysis are counted or real time PCR is done in order to determine recovery efficiency. However, these methods are time consuming and do not allow determination of the recovery efficiency of all virus. It only evaluates the recovery rate of the added virus. Moreover, most viruses cannot be cultured and, consequently, cannot be detected by lysis plaques. The enumeration of the virus-like particles (VLP), by epifluorescence, can be a simple, fast and cost-effective approach to determine viral recovery. The number of natural virus present in the water sample can be determined before and after sample concentration, allowing evaluation of the concentration of all viruses present in the sample. Although this technique has been frequently used to determine the viral abundance in aquatic systems (Shields anf Farrah, 1986; Almeida et al, 2001; Danovaro et al, 2002; Suttle and Fuhrman, 2010) it was never used to evaluate the efficiency of viral recovery of concentration methods.

The objective of this work was to evaluate ultracentrifugation as the main method to directly concentrate viruses in environmental waters, using a simple and rapid approach (determination of the VLP number) to determine the efficiency of viral recovery.

As the flocculation method is normally employed to concentrate viruses in environmental waters (Guttman-Bass and Armon, 1983; Guttman-Bass and Nassen, 1984; Shields and Farrah, 1986; Calgua et al, 2008; virobathe, 2011), it was also used in this study to assess the efficacy of ultracentrifugation as the main concentration method in environmental waters.

Material and Methods

Water sampling

Wastewater and recreational water samples were tested. Wastewater samples were collected at a wastewater treatment plant of Aveiro (South ETAR) after secondary treatment and recreational water samples were collected in a brackish water zone of Ria de Aveiro. Both wastewater and recreational water were collected twice between March and August 2009. Samples of 1 L for sewage treated water and of 10 L for recreational water were used for the direct flocculation method. For the ultracentrifugation method, sub-samples of 0.5 L for sewage treated water and of 1 L for recreational water were used. Three independent assays were done for each situation at each sampling date.

Virus concentration by flocculation

The flocculation method used was based on the protocol described by Calgua et al, 2008. Water samples were acidified with HCl to pH 3.5 (0.1) and added to 50 mL (for sewage

treated water) or 100 mL (for recreational water) of skim milk solution (1% w/v) at pH 3.5 (±0.1). Samples were slowly stirred with a magnetic stirrer for 10 h at room temperature and then flocs were allowed to sediment by gravity for 8 to 10 h. Supernatants were carefully removed without disturbing the sediment and the final volume (approximately 500 mL) was centrifuged at 7000 x g for 30 min at 4ºC. Supernatant was carefully removed and the pellet was suspended in 8 mL of 1x PBS. pH was adjusted to 7.5 (±0.1) by the addition of 1 M NaOH and 1x PBS was added to a final volume of 10 mL. Concentrated samples were stored at -80 ºC.

Virus concentration by ultracentrifugation

Each water sample was filtered with 0.2 µm membranes (142 mm ø; Millipore Durapore) at low pressure (<200 mm Hg) using a filter system (A.E.B., S.R.L. Druck Ablassen, Italia) and then centrifuged (Beckman Optima™, LE-80 K Ultracentrifuge, rotor 50.2 Ti) at 100 000g for 1 h at 20 ºC. Supernatant was removed and the pellet was suspended in 1 mL of 1x PBS. Final centrifugation was done at 100 000g for 1 h at 20ºC to gather the pellet. Supernatant was removed and the pellet was suspended in 200 µL of 1x PBS. Concentrated samples were stored at -80 ºC.

Determination of the efficiency of recovery

The efficiency of recovery was determined by counting VLP before and after sample concentration by flocculation and ultracentrifugation by the epifluorescence microscopy method using a modified method of Noble and Fuhrman, 1998. Water samples were filtered with a 0.2 µm polycarbonate membrane and then with a 0.02 mmAl2O3 Anodisc, which were then stained in the dark for 20 min with SYBR gold dye (0.25%). Enumeration of VLP was made using a Leitz Laborlux K epifluorescence microscope. For each sub-sample, 3 replicates were observed and at least 200 virus were counted in each replicate. Three independent assays were done for each situation of each sampling date.

Detection and quantification of enteric virus in concentrated water samples by PCR and qPCR

Both virus, adenovirus and rotavirus, were detected in the concentrated samples (by flocculation and ultracentrifugation) by PCR in wastewater and in recreational waters, but only the adenovirus were quantified by qPCR for concentrated (by flocculation and ultracentrifugation) recreational waters. Adenovirus were chosen for quantification because this viral group has been suggested as a potential indicator of the presence of viral pollution in environmental waters, namely in marine waters (Fong and Lipp, 2005;

Muscillo et al, 2008; Albinana-Gimenez et al, 2009). Three independent assays were done for each situation in each sampling date.

Nucleic acids were extracted from water samples using the geneMAG-RNA/DNA kit, a magnetic RNA/DNA purification kit (Chemicell™), according to the instructions of the manufacturer.

For nucleic acid purification the Geneclean kit (MP Biomedicals, LLC) was used according to the instructions of the manufacturer.

Detection of adenovirus and rotavirus was performed in a Labnet TC9600-G thermocycler and amplification products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and detected with a UV transilluminator.

Detection of Adenovirus

Detection of adenovirus was made by a nested PCR technique using the primers described by Allard et al, 2001. Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2 mM Buffer taq, 1.5 mM MgCl2, 1.28 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.4 mM of each primer (StabVida) (**hex1deg** 50-GCC SCA RTG GKC WTA CAT GCA CAT C-30 and **hex2deg** 5'-CAG CAC SCC ICG RAT GTC AAA-3'). After the first PCR, 5 µL of PCR product were added to 20 µL of a new reaction mixture consisting of 1 mM Buffer taq, 1.5 mM MgCl2, 1.28 mM dNTPs, 0.4 mM of each primer (**nehex3deg** 5' GCC CGY GCM ACI GAI ACS TAC TTC 3' and **nehex4deg** 5' CCY ACR GCC AGI GTR WAI CGM RCY TTG TA 3') and 0.1 U μmL^{-1} of Taq polymerase.

All primers sequences are found between base pair position 21 and position 322 in the coding region of the hexon gene. The first set of primers creates a 301-bp product and the second set of primers creates a 171-bp product.

The amplification was carried out for 45 cycles at 94ºC for 10 s, 55ºC for 30 s and 72C for 20 s after initial denaturation at 94ºC for 4 min. A final extension step was performed at 72ºC for 5 min. The amplification products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and detected with a UV transilluminator.

Detection of Rotavirus

Detection of rotavirus was made with a PCR technique using the primers described by Villena et al, 2003 and using the Super- Script™ II RT for the synthesis of cDNA, according to the manufacturer instructions. Five microliters of sample were added to 20 µl of the reaction mixture, which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U μmL^{-1} of Taq polymerase (Fermentas) and 0.48 mM of each primer

(StabVida) (**VP6-3** 5'-GCT TTA AAA CGA AGT CTT CAA C-3' and **VP6-4** 5'-GGT AAA TTA CCA ATT CTT CCA G-3).
Rotavirus primers positions is found between the position 187 and position 166 of human strain Wa [accession number K02086]), creating a product of 186-bp.
The amplification was carried out for 40 cycles at 94ºC for 10 s, 50ºC for 30 s and 72ºC for 20 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.
The amplification products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and detected with a UV transilluminator.

Quantification of Adenovirus in concentrated water samples by qPCR
The quantification of adenovirus was performed in an iQ5 thermocycler and standards were obtained from a serial dilution of a suspension of Adenovirus, with a known initial number of copies (obtained by nucleic acid quantification with nanodrop). The qPCR was performed using the same protocol as that described for detection of Adenovirus using the PCR technique, but with a reaction mixture of 2x iQ™ SYBR™ Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6 mM MgCl2, SYBR Green I, fluorescein and stabilizers) and 0.4 mM of each primer (nehex3deg and nehex4deg), to which was added 5 µl of sample.

Statistical analysis
For the enumeration of virus-like particles (VLP) and for the detection and quantification of the two enteric viruses, three sub-samples were used for both concentration methods and for both water types of each sampling date. For the enumeration of VLP, for each sub-sample, 3 replicates were analysed and for the detection and quantification of the enteric virus only 2 replicates were done for each sub-sample. The results of the three sub samples were averaged and the standard deviation was calculated.
The differences between the efficiency of recovery, determined by the enumeration of VLP, of the two concentration methods were analyzed by one-way ANOVA to check for significant differences between methods. The difference between Adenovirus quantification in recreational waters concentrated by the two methods was also evaluated using one-way ANOVA. Only the data with normal distribution (assessed by the Kolmogorov–Smirnov test) and with homogeneity of variances (assessed by Levene's test) were used. A value of $p < 0.05$ was considered significant. Statistical analyses were performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA).

Results

Efficiency of viral recovery for the two concentration methods

The recovery efficiency for sewage samples (Fig. 2.1a) and recreational samples (Fig. 2.1b) was higher with the ultracentrifugation than with the flocculation method. Ultracentrifugation showed average recovery efficiency of 69% for wastewater and of 76% for recreational water samples. The corresponding recovery efficiencies with the flocculation method were 38% and 22% for wastewater and recreational water, respectively.

The differences between the efficiency of recovery for the two sampling dates were not significant for wastewater ($p = 0.86$ and $p = 0.16$ respectively), but for recreational waters there were significant differences in the efficiency of recovery with the two concentration methods for both sampling dates ($p = 0.01$ and $p = 0.00$, respectively).

Detection of enteric viruses in water samples

The adenovirus and rotavirus A in wastewater and recreational water samples were found in all samples after concentration by ultracentrifugation and by flocculation.

Quantification of Adenovirus in recreational water samples

The presence of adenovirus in recreational water samples after concentration was tested by qPCR (Fig. 2.2). This group of virus was present in all samples after concentration.

The differences between the quantification of adenovirus, for the two concentration methods, for the two sampling dates, were not significant ($p = 0.057$ and $p = 0.868$, respectively).

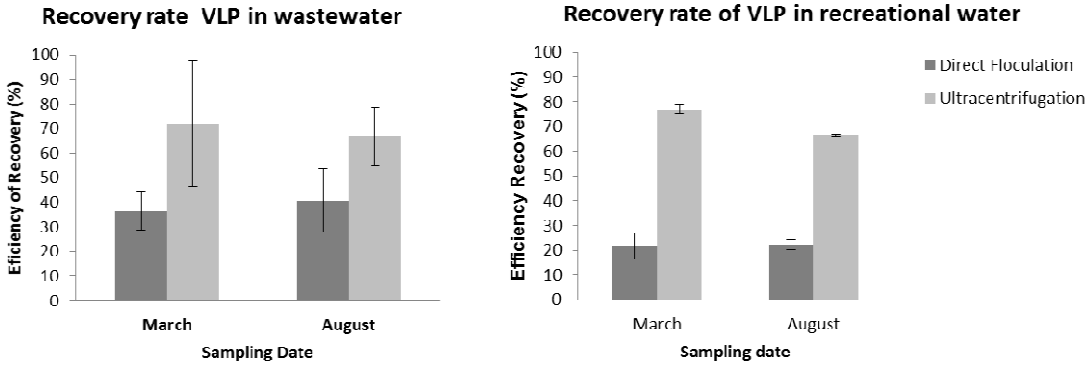

Fig. 2.1: (a) Recovery rate of VLP, for both concentration methods, in sewage waters, for two sampling dates. (b): Recovery rate of VLP, for both concentration methods, in recreational waters, for two sampling dates. Values represent the mean of three independent experiments; error bars represent the standard deviation.

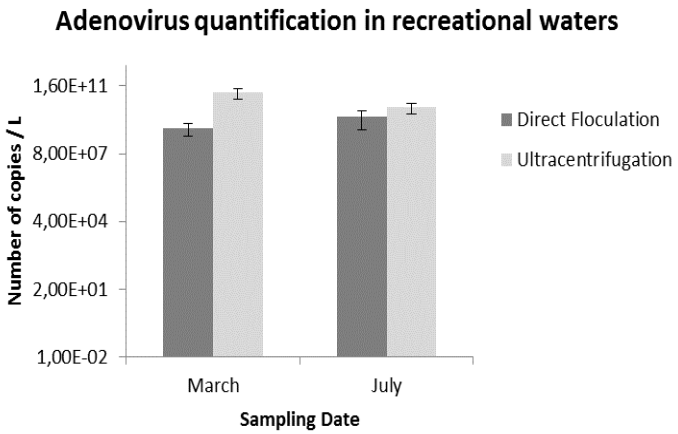

Fig. 2.2: Quantification of adenovirus in recreational water samples after concentration by flocculation and ultracentrifugation for the two sampling dates. Values represent the mean of three independent experiments; error bars indicate the standard deviation.

Discussion

Environmental samples must be sufficiently concentrated to allow efficient detection of very few viruses in a large volume of water (Schwab et al, 1995). However, there is no perfect viral concentration method for water samples (Bosch et al, 2008; Albinana-Gimenez et al, 2009), the method should be chosen so that, despite its limitations, it is adequate to achieve the aim of the work. In the present work, the ultracentrifugation and the flocculation methods were compared, considering further use of the samples for molecular analysis such as PCR or pyrosequencing. The results of this study show that the ultracentrifugation method, usually used as a secondary step for virus concentration (Steward, 2001; Fout et al, 2003; Kovac et al, 2009; Sylvain et al, 2009), is an adequate approach to concentrate virus directly from environmental waters and that the determination of the VLP by microscopy is a simple, fast and cost effective method to evaluate the recovery efficiency of the concentration method.

The ultracentrifugation method recovered about 30% more viruses in residual waters and about 50% more in recreational waters than the organic flocculation method, one of the most currently used approaches to concentrate viruses from environmental waters (Calgua et al, 2008; Guttman-Bass and Armon, 1983; Guttman-Bass and Nassen, 1984; Shields and Farrah, 1986; virobathe, 2011). Moreover the ultracentrifugation method is simpler and faster than the flocculation approach.

During flocculation, HCl is added to water samples to adjust the water pH to 3.5 and skim milk solution is added to allow virus adsorption. It is, however, well known that during the

concentration step, inhibitory substances are concentrated along with the virus and, consequently, the addition of HCl and skim milk solution may increase the inhibitory effect on subsequent PCR reactions. In ultracentrifugation, only the non-added inhibitory substances are concentrated. Although for recreational water this effect can be negligible, for wastewaters, which have large concentrations of inhibitory substances, purification of the concentrated samples is always required (Guo et al, 2009; Moussani et al, 2009). For more clean environmental waters this can be an inconvenience relative to the ultracentrifugation method. The incubation period of more than 16 hours used for flocculation may also affect the structure of the viral community. It is well known that bacteriophages represent a large fraction of the virioplankton (Bettarel et al, 2008; Miki and Jacquet, 2008; Suttle and Fuhrman, 2010) and their replication cycle is frequently less than 1 hour (Bettarel et al, 2008; Madigan and Martinko, 2006), allowing these virus to undergo several replications during the period of concentration by flocculation. In contrast, viruses that infect eukaryotic cells have replication times of around 40 hours and, consequently, it is unlikely that they replicate during the incubation period. These facts are not important if the concentration method is used to detect specific enteric virus by PCR because specific primers are used, but if the concentrated water samples are used to study the structure of the viral community, the results will not reflect the structure of the original community. In contrast, in the ultracentrifugation method, as water samples are filtered with 0.2 mm membranes in order to remove bacteria prior to centrifugation, all cellular hosts are removed and viral replication is avoided, even for bacteriophages that have shorter life cycles. However, the water filtration before ultracentrifugation can cause viral loss due to membrane clogging. The replacement and/or the employment of large-size membranes, as used in this study, can overcome this problem.

Moreover, when ultracentrifugation is used to detect specific enteric viruses, water filtration is not necessary and, consequently the loss of virus by membrane clogging is avoided. Therefore, the concentration by ultracentrifugation provides a more realistic picture of the viral community structure than the flocculation method.

Although it is not practical to ultracentrifuge large volumes of water, it is possible to reduce the final volume of concentrated samples to a few microlitres and, consequently, efficiently concentrate virus in environmental waters. In this study, water samples of 0.5–1.0 L were concentrated 1000–2000 times in a volume of 200 mL, while by flocculation water samples of 10 L were concentrated 100–1000 times to a final volume of 10 mL. The large amount of flocs formed during the precipitation with the skim milk prevents reduction of the concentrated water volume of the flocculated samples to less than 10 mL. Moreover, techniques of nucleic acid amplification are now the most common way for viral detection

in water since they are rapid and more sensitive than traditional cell culture methods (Hovi et al, 2007; Pinto et al, 2007; Rodriguez-Diaz et al, 2009). Consequently, a small volume of water is sufficient to detect a specific virus. In fact, in this study enteric viruses were detected by PCR in recreational waters at sites where, in previous studies, their presence had been undetectable by cell culture and immunological methods (Alcântara and Almeida, 1995).

The results show that both flocculation and ultracentrifugation methods are adequate to concentrate water samples for detection and quantification of enteric viruses in environmental waters. rotavirus A and adenovirus were present in all samples concentrated by either of the two concentration methods and the number of Adenovirus in recreational waters, quantified by qPCR, was similar for both concentration methods in the three independent assays of each of the two sampling dates. The estimation of the recovery efficiency based on the enumeration of VLP by epifluorescence was similar to that achieved in other studies based on the addition of specific enteric viruses to water samples and further quantification by plaque assay (Guttman-Bass and Nassen, 1984; Shields and Farrah, 1986; Haramoto et al, 2005; Kovac et al, 2009) or qPCR (Calgua et al, 2008; Muscillo et al, 2008; Hamza et al, 2009; Kovac et al, 2009). The enumeration of VLP by epifluorescence is, however, a simpler, faster and cheaper approach relative to the traditional plaque assay and qPCR approaches. Moreover, using the VLP, it is possible to evaluate the concentration of the whole viral community and not of a specific virus, as it happens when the viruses are added to the samples and detected by plaque assay and PCR techniques. In contrast to the plaque assay enumeration, using VLP counts it is considered the infective and non-infective viruses. However, since the techniques of nucleic acid amplification are now the most common way for viral detection/quantification in environmental waters (Tsai et al, 1993; Villena et al, 2003; Hovi et al, 2007; Pinto et al, 2007) and these techniques amplify nucleic acids of both infective and non-infective viruses, the recovery efficiency based on the enumeration of VLP is not an inconvenience, reflecting even better the efficacy of the concentration methods to recover viruses in environmental waters. Consequently, the determination of VLP is a good alternative to determine the recovery efficiency of the viral concentration methods.

Acknowledgements

The authors would like to thank the University of Aveiro and Centre for Environmental and Marine Studies (CESAM) for funding the study. Financial support to C. Prata was provided by the Portuguese Foundation for Science and Technology (FCT) in the form of a PhD grant (SFRH/BD/33390/2008), São José Nascimento of University of Porto and Albert Bosh of University of Barcelona, for providing viral stocks used in this work as positive controls.

References

- Albinana-Gimenez N, Clemente-Casares P, Bofill-Mas S, Hundesa A, Ribas F, Girones R, 2006, Distribution of human polyomaviruses, Adenoviruses and hepatitis E virus in the environmental and drinking-water treatment plant, Environmental Science and Technology, 40, 7416–7422.
- Albinana-Gimenez N, Miagostovich M P, Calgua B, Huguet J M, Matia L, Girones R, 2009, Analysis of Adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants, Water Research, 43, 2011–2019.
- Alcântara F, Almeida M A, 1995, Virological quality of the Ria de Aveiro: validity of potential microbial indicators, Aquatic Ecology, 29(3–4), 419–425.
- Allard A, Albisson B, Wadell G, 2001, Rapid Typing of Human Adenoviruses by a General PCR combined with Restriction Endonuclease Analysis, Journal of Clinical Microbiology, 39 (2) 498–505
- Almeida M A, Cunha M A, Alcântara F., 2001, Loss of estuarine bacteria by viral infection and predation in microcosm conditions, Microbial Ecology, 42(4), 562–571.
- Angly F E, Felts B, Breitbart M, Salamon P, Edwards R A, Chan A M, Haynes M, Kelley S, Liu H, Mahaffy J M, Carlson C, Mueller J E, Nulton J, Olson R, Parsons R, Rayhawk S, Suttle C. A, Rohwer F, 2006, The Marine Viromes of Four Oceanic Regions, PLoS Biol, 4(11: e368.
- Bettarel Y, Arfi R, Bouvier T B, Bouvy M, Briand E, Colombet J, Corbin D, Sime-Ngando T, 2008, Virioplankton distribution and activity in a tropical eutrophicated bay, Estuarine, Coastal Shelf Sci., 80, 425–429.
- Block J C, Schwartzbrod L, 1989, Viruses in Water Systems: Detection and Identification, VCH Publishers, Inc, New York.
- Bosch A, 1998, Human enteric viruses in the water environment: a minireview, Int. Microbiology, 1, 191–196.

- Bosch A, Guix S, Sano D, Pinto R M, 2008, New tools for the study and direct surveillance of viral pathogens in water, Current Opinion in Biotechnology, 19, 295–301.
- Branovic K, Forcic D, Ivancic J, Strancar A, Barut M, Kosutic- Gulija T, Zgorelec R, Mazuran R, 2003, Application of short monolithic columns for improved detection of virus, Journal of Virological Methods, 110, 163–171.
- Caballero S, Guix S, El Senousy W M, Calico I, Pintó R M, Bosch A, 2003, Persistent gastroenteritis in children infected with Astrovirus: association with serotype-3 strains, Journal of Medical Virology, 71, 245–250.
- Calgua B, Mengewein A, Grunert A, Bofill-Mas S, Clemente- Casares P, Hundesa A, Wyn-Jones A P, Lopez-Pila J M, Girones R, 2008, Development and application of a one-step low cost procedure to concentrate viruses from seawater samples, Journal of Virological Methods, 153, 79–83.
- Costafreda M I, Bosch A, Pintó R M.,2006, Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples, Applied and Environmental Microbiology, 72(6), 3846–3855.
- Danovaro R, Manini E, Dell'Anno A, 2002, Higher abundance of bacteria than virus in deep Mediterranean sediments, Applied and Environmental Microbiology, 68, 1468–1472.
- Fong T-T, Lipp E K, 2005, Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools, Microbiology and Molecular Biology reviews, 69 (2) 357-371.
- Fout G S, Martinson B C, Moyer M W N, Dahling D R, 2003, A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater, Applied and Environmental Microbioligy, 69 (6), 3158–3164.
- Garrett W D, 1965, Collection of slick-forming materials from the sea surface, Limnology and Oceanography, 10, 602–605.
- Grassi T, Bagordo F, Ídolo A, Lugoli F, 2009, Rotavirus detection in environmental water samples by tangential flow ultrafiltration and RT-nested PCR, Environmental Monit Assessment.
- Guo H, Chu X, Hu J, 2010, Effect of Host Cells on Low- and Medium-Pressure UV Inactivation of Adenoviruses, Applied and Environmental Microbiology, 76 (21) 7068–7075.

- Gutierez-Aguirre I, Banjac M, Steyer A, Poljsak-Prijatelj M, Peterka M, Strancar A, Ravnikar M, 2008, Concentrating rotavirus from water samples using monolithic chromatographic supports, Journal of Chromatography, 1216, 2700–2704.
- Guttman-Bass N, Armon R, 1983, Concentration of Simian Rotavirus SA-11 from tap water by membrane filtration and organic flocculation, Applied and Environmental Microbiology, 45(3), 850– 855.
- Guttman-Bass N, Nassen A, 1984, Simultaneous concentration of four enterovirus from tap, waste and natural waters, Applied and Environmental Microbiology, 47 (6), 1311–1315.
- Hamza I A, Jurzik L, Stang A, Sure K, Uberla K, Wilhelm M, 2009, Detection of human viruses in rivers of a densely-populated area in Germany using a virus adsorption method optimized for PCR analyses, Water Research, 43, 2657–2668.
- Haramoto E, Katayama H, Oguma K, Ohgaki S, 2005, Application of cation-coated filter method to detection of norovirus, enterovirus, Adenovirus, and torque teno virus in the Tamagawa river in Japan, Applied and Environmental Microbiology, 71(5), 2403–2411.
- Hovi T, Roivainen M, Blomqvist S, 2007, Enterovirus with Special Reference to Poliovirus and Poliomyelitis Eradication, Human Virus in Water: Perspectives in Medical, 69–89.
- Hsu B-M, Chen C-H, Kung C-M, Wan M-T, Shen S-M, 2007, Evaluation of enterovirus recovery in surface water by different adsorption and elution procedures, Chemosphere, 66, 964–969.
- Katamaya H, Shimasaki A, Ohgaki S, 2002, Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater, Applied and Environmental Microbiology, 68(3), 1033–1039.
- Katzenelson E, Fattal B, Hostovesky T, 1976, Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water, Applied and Environmental Microbiology, 32(4), 638–639.
- Kovac K, Gutierrez-Aguirre I, Banjac M, Peterka M, Prijatelj M P, Ravnikar M, Mijovski J Z, Schultze A C, Raspor P, 2009, A novel method for concentrating hepatitis A virus and caliciviruses from bottled water, Journal of Virological Methods, 162, 272–275.
- Kramberger P, Petrovic N, Strancar A, Ravnikar M, 2004, Concentration of plant virus using monolithic chromatographic supports, Journal of Virological Methods, 120, 51–57.

- LaBelle R L, Gerba C P, Goyal S M, Melnick J L, Cech I, Bogdan G F,1980, Relationship between environmental factors, bacterial indicators, and the occurrence of enteric virus in estuarine sediments, Applied and Environmental Microbiology, 39(3), 588–596.
- Lambertini E, Spencer S K, Bertz P D, Loge F J, Kieke B A, Borchardt M A, 2008, Concentration of enterovirus, Adenovirus, and norovirus from drinking water by use of glass wool filters, Applied and Environmental Microbiology, 74(10), 2990–2996.
- Lee S H, Kim S J, 2002, Detection of infectious enteroviruses and Adenoviruses in tap water in urban areas in Korea, Water Research, 36(1), 248–256.
- Madigan M, Brock M J, 2006, Biology of Microorganisms, Pearson Prentice Hall, Inc.
- Miki T, Jacquet S, 2008, Complex interactions in the microbial world: under-explored key links between virus, bacteria and protozoan grazers in aquatic environments. A Review, Aquatic Microbial Ecology, 51, 195–208.
- Moussani G, Mahmoudi M, Barikbin B, 2009, Biological removal of phenol from strong wastewaters using a novel MSBR, Water Research, 43, 1295–1302.
- Muscillo M, Pourshaban M, Iaconelli M, Fontana S, Di Grazia A, Manzara S, Fadda G, Santangelo R, La Rosa G, 2008, Detection and quantification of human Adenoviruses in surface waters by nested PCR, TaqMan Real-Time PCR and cell culture assays, Water, Air, Soil Pollution, 191, 83–93.
- Myrmel M, Berg E M M, Grinde B, Rimstad E, 2006, Enteric viruses in inlet and outlet samples from sewage treatment plants, Journal of Water Health, 197–209.
- Noble R T, Fuhrman J A, 1998, Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria, Aquatic Microbial Ecology, 14, 113–118.
- Ozawa K, Oka T, Takeda N, Hansman G S, 2007, Norovirus infections in symptomatic and asymptomatic food handlers in Japan, Journal of Clinical Microbiology, 45(12), 3996–4005.
- Percival S, Chalmers R, Embrey M, Hunter P, Sellwod J, Wyn-Jones P, 2004, Microbiology of Waterborne Diseases, Viruses, Elsevier Academic Press, London, 1st edn, pp. 337–452.
- Pinto R M, Alegre D, Dominguez A, El Senousy W M, Sanchez G, Villena C, Costafreda M I, Aragones L, Bosch A, 2007, Hepatitis A virus in urban sewage from two Mediterranean countries, Epidemiology and Infections, 135, 270–273.

- Polaczyck A L, Roberts J M, Hill V R, 2007, Evaluation of 1MDS electropositive microfilters to simultaneous recovery of multiple microbe classes from tap water, Journal of Microbiological Methods, 68, 260–266.
- Puig M, Jofre J, Lucena F, Allard A, Wadell G, Girones R, 1994, Detection of Adenoviruses and enteroviruses in polluted water by nested PCR amplification, Applied and Environmental Microbiology, 60(8), 2963–2970.
- Rodriguez-Diaz J, Querales L, Caraballo L, Vizzi E, Liprandi F, Takiff H, Betancourt W Q, 2009, Detection and characterization of waterborne gastroenteritis viruses in urban sewage and sewagepolluted river waters in Caracas, Venezuela, Applied and Environmental Microbiology, 75(2), 387–394.
- Schwab K J, De Leon R, Sobsey M, 1995, Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A viruses, and norwalk viruses by reverse transcription-PCR, Applied and Environmental Microbiology, 61 (2) 527–531.
- Sedmak G, Bina D, Macdonald J, Couillard L, 2005, Nine-year study of the occurrence of culturable viruses in source water for two drinking water treatment plants and the influent and effluent of a wastewater-treatment plant in Milwakee, Wisconsin, Applied and Environmental Microbiology, 71, 1042–1050.
- Shields P A, Farrah S R, 1986, Concentration of viruses in beef extract by flocculation with ammonium sulfate, Applied and Environmental Microbiology, 51(1) 211–213.
- Steward G F, 2001, Fingerprinting viral assemblages by pulsed Field gel electrophoresis (PFGE), Methods Microbiology, 30.
- Suttle C A, Fuhrman J A, 2010, Enumeration of Virus Particles in Aquatic or Sediment Samples by Epifluorescence Microscopy, in Manual of Aquatic Viral Ecology, ed. S. W. Wilhelm, M. G. Weinbauer and C. A. Suttle, ASLO, 145–153.
- Sylvain S, Gantzer C, Helmi K, Hoffmann L, Cauchie H-M, 2009, Simultaneous concentration of enteric virus and protozoan Parasites: a protocol based on tangential flow filtration and adapted to large volumes of surface and drinking waters, Food and Environmental Virology, 1(2), 66–76.
- Tsai Y L, Sobsey M D, Sangermano L R, Palmer C J, 1993, Simple Method of Concentrating Enteroviruses and Hepatitis A Virus from Sewage and Ocean Water for Rapid Detection by Reverse Transcriptase-Polymerase Chain Reaction, Applied and Environmental Microbiology, 59 (10), 3488-3491.

- Victoria J G, Kapoor A, Li L, Blinkova O, Slikas B, Wang C, Naeem A, Zaidi S, Delwart E, 2009, Metagenomic analysis of viruses in stool samples from children with acute flaccid paralisys, Journal of Virology, 83(9) 4542–4651.
- Villena C, El-Senousy W M, Abad F X, Pinto R M, Bosch A, 2003, Group A rotavirus in sewage samples from Barcelona and Cairo: emergence of unusual genotypes, Applied and Environmental Microbiology, 69 (7) 3919–3923.
- Virobathe, www.virobathe.org, 2011.
- Wommack K E, Colwell R R, 2000, Virioplankton: virus in aquatic ecosystems, Microbiol. Mol. Biol. Rev., 64(1) 69–114.

Chapter 3 - Surface microlayer as a source of health risk relevant enteric viruses in Ria de Aveiro

Surface microlayer as a source of health risk relevant enteric viruses in Ria de Aveiro

Catarina Prata, Ângela Cunha, Newton Gomes, Adelaide Almeida

Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

Abstract

The sea surface microlayer (SML) corresponds to the interface between the surface of the water and the air and can accumulate microbial particles, such as viruses. While some studies are already focused on the concentration of viruses in the SML, the potential of this layer to concentrate health risk relevant enteric viruses has never been evaluated. However, information about enteric viruses in the SML is needed in order to evaluate its role as a source of significant health risk viruses and their interaction with atmosphere. In this work the presence of three enteric viruses (rotavirus, enterovirus and HAV) was detected and the abundance was determined in SML and underwater (UW) in Ria de Aveiro. All groups were detected in the SML and the abundance of rotavirus, enterovirus and HAV was higher in the SML than in UW (enrichment factors ranging from 1.01 to 6.37 in the marine zone and from 1.12 to 1.53 in the brackish water zone for rotavirus, from 1.01 to 2.80 in the marine zone and from 1.03 to 5.05 in the brackish water zone for enterovirus and from 1.02 to 2.15 in the marine zone and from 1.17 to 1.44 in the brackish water zone for HAV). The enteroviruses and rotaviruses abundance variation was explained by the studied physico-chemical parameters, namely water temperature and nitrates concentration but the variation of HAV was not explained by any of the studied physico-chemical parameters. The results indicate, for the first time, that enteric viruses, which seem to have mainly terrestrial origin, can be concentrated at the SML, reaching frequently values around two times higher than those of the UW, which suggests that SML can act as a source of enteric viruses in the aquatic environment.

Introduction

Health relevant enteric viral groups are considered to be waterborne pathogens (Noble and Fuhrman, 1998; Sedmak *et al*, 2005; Albinana -Gimenez *et al*, 2006; Myrmel *et al*, 2006), increasing the concern over the discharge of human enteric viruses into not only freshwater but also into estuarine and marine environments (Lee and Kim, 2002; Hamza *et al*., 2009; Rodríguez-Díaz *et al*, 2009).

This group of viruses is stable in water and can exist naturally in the aquatic environment or could be transported to this environment through sewage outfall and vessel wastewater discharge (Grabow 1996, Pianetti *et al*. 2000, Griffin *et al.* 2003, Suttle 2005, Bosh *et al*. 2006, Suttle 2007, Lugoli *et al*. 2009). Although viruses can survive in water, as they are obligate intracellular parasites they cannot multiply in the environment (Wyn-Jones and Sellwood 2001). However, they can maintain infectivity, even after long periods in the environment, surviving for more than 130 days in seawater at temperatures between 20 and 30ºC (Rzezutka & Cook, 2004), increasing the probability of human exposure by recreational contact (Fong and Lipp, 2005).

Although it is well known that viruses are of particular concern to human health, scarce information about their natural reservoirs, such as surface microlayer (SML) is available. Despite some studies on infectivity, survival, abundance, diversity and interactions of viruses with their hosts in the SML (Weinbauer 2004), showing that these are as abundant as in the underwater (UW) (Parada et al, 2005), until now, there is no study on human pathogenic virus in the SML.

The sea SML is generally defined as the uppermost millimeter of the ocean (Liss and Duce 1997) and corresponds to the interface between the surface of the water and the air, forming a physically stable environment, but climatically variable. Water column processes regulate the accumulation of material in the SML and so, small molecules and larger particles can accumulate to form a film that extends into the UW (Cunliffe et al, 2011). The location of the SML makes it a highly dynamic system (Cunliffe et al, 2011) and in comparison with the UW, SML is physically more stable due to surface tension (Hardy, 1982) and the presence of a biogenic gelatinous film layer, constituting a microbial habitat where compounds and particles can be concentrated (Cunliffe et al, 2011). Therefore, the SML is generally enriched in organic materials, which might stimulate biological growth. However, it has been shown that organism density, activity and diversity in the SML can be higher, similar or lower that in UW. As SML interact with both the atmosphere and the hydrosphere simultaneously (Cunliffe et al, 2011), this layer may play an important role in microbial concentration and transmission across the air-water interface, acting as a reservoir for microorganisms, such as enteric viruses, in the aquatic environment.

The aim of this study was to evaluate the role of SML as a source of three groups of enteric viruses (rotavirus, enterovirus and HAV) in the aquatic environment.

Material and methods

Study area and sample collection

Ria de Aveiro is a branched estuarine ecosystem, located in the northwest coast of Portugal, connected to the Atlantic by a narrow opening and separated from the sea by a sand bar. The system covers an area of 66 to 83 km^2 at low and high tide, respectively (Silva, 1994; Dias et al, 2000).

Water samples were collected in a marine zone (MZ) (N1), in Canal de Navegação and in a brackish water zone (BWZ) (I6), in Canal de Ilhavo, both in Ria de Aveiro (Figure 3.1) in four dates of 2010 (two in the cold season (January and February) and two in the warm season (June and July)). In each sampling date, three independent samples were collected at the SML and UW.

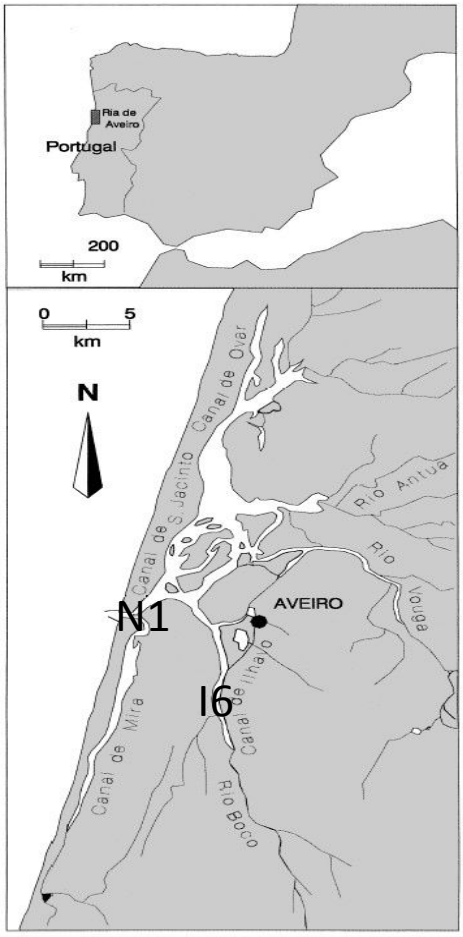

Figure 3.1: The estuarine system Ria de Aveiro with an indication of the sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ilhavo, represents the brackish water zone.

SML samples were collected with plexiglass and glass plates (0.25 m wide, 0.35 m long, and 4 mm thick). Prior to sample collection, the plates were rinsed with ethanol and sterile distilled water. The plates were introduced vertically through the SML and withdrawn in the same position. The excess of water was allowed to drain for about 5 s. Approximately 5 mL of water were collected each time the plate was introduced into the SML. The water adhering to the plate was subsequently removed from both sides with a wiper blade system (Harvey and Burzell 1972). The estimated thickness of the collected SML, determined from the volume of collected sample and the area of both sides of the plate, was approximately 60 µm, which is in the range reported by Harvey and Burzell (1972) for glass plate samplers. For SML samples, 2.5 L were collected.

For UW samples, 5 L were collected by submerging a sterilized glass bottle 15 cm in the water column. Three samples of SML and of UW were collected in each date. Water samples were maintained at 4 °C until analysis.

Physical and chemical variables

Phisical parameters were only determined for UW, because the collection for the SML is a labourious process during which changes in water temperature and salinity may occur. Water temperature and salinity were determined in the field with a WTW (Wissenschaftlich TechnischeWerkst¨atten, Germany) Cond330i/SET.

The concentration of suspended solids (seston) was performed after filtration of aliquots of 500 mL for UW and of 150 mL for SML through Whatman GF/F (47 mm diam.) pre-weighted, pre-combusted filters. The filters were dried at 60°C for 24 h and seston content was calculated as the increase in weight (Parsons et al. 1989).

For each nutrient, three replicates of 5 mL were filtered through GF/F Whatman filters (47 mm diameter) and frozen for later analysis. Ammonium (NH_4^+) was quantified following the indophenol blue method (Dias et al, 2000). Nitrates (including nitrites) were determined by the sulfanilic acid method after reduction of nitrates to nitrites in a cadmium column (Lorezen, 1967). Phosphates were determined by the molibdate blue method (Koroleff, 1970).

Chlorophyll a was determined espectrophotometrically after extraction by 90% acetone (Yentsch & Menzel 1963). Three replicates of 500 mL and three replicates of 150 mL were filtered for UW and SML, respectively, with GF/F Whatman filters. The filters were stored at -20ºC, until analysis, with 10 mL acetone 90%. A standard curve was determined, at an emission wavelength of 660 nm and 440 nm for excitation wavelength.

Water samples concentration

Water samples were concentrated, using the ultracentrifugation method, as described by Prata et al (2012). Samples were centrifuged (Beckman Optima™, LE-80K Ultracentrifuge, rotor 50.2 Ti) at 100000 g for 1 hour at 20°C and the pellet was ressuspended in 200 µl of 1x PBS.

Nucleic acids extraction

Nucleic acids were extracted from concentrated water samples from SML and UW, using the geneMAG-RNA/DNA kit, a magnetic RNA/DNA purification kit (Chemicell ™), according to the instructions of the manufacturer. cDNA synthesis was made using a NZY

First-Strand cDNA Synthesis Kit (Nzytech), using the forward primers described below in the PCR technique. The nucleic acids were stored at -80°C until analysis.

Enteric virus detection and quantification

All viral groups were detected using a TPersonal thermocycler (Biometra) for PCR technique. The amplification products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and detected with UV transilumination.

The quantification was made, using a stepone plus 46 weel thermocycler (Applied Biosystems). Standards were obtained from a serial dilution of a suspension of each viral group, with a known initial number of copies, obtained by nucleic acid quantification with nanodrop. Negative controls were made, by using sterilized miliQ-water, instead of nucleic acids.

Detection of rotavirus

Detection of rotavirus was made by a PCR technique using the primers described by Villena et al, 2003. Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.48 mM of each primer (StabVida) (**VP6-3** 5': GCT TTA AAA CGA AGT CTT CAA C: 3' and **VP6-4** 5': GGT AAA TTA CCA ATT CTT CCA G: 3'). Rotavirus primers position is found between the positions 187 and 166 of human strain Wa (accession number K02086), creating a fragment of 186-bp.

The amplification was carried out for 40 cycles of 94ºC for 10 s, 50ºC for 30 s and 72ºC for 20 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Detection of enterovirus

Detection of enterovirus was made by a PCR technique using the primers described by by Beld et al, 2004. Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.48 mM of each primer (StabVida) (**Entero 1** 5'- CCC TGA ATG CGG CTA AT -3' and **Entero 2** 5'- ATT GTC ACC ATA AGC AGC CA - 3'). Enterovirus primers sequences used for amplification are located in the conserved 5' noncoding region of the EV sequence. Nucleotide positions 452 to 468 for Entero-1 and 579 to 597 for Entero-2, creating a fragment of 172-bp.

The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Detection of HAV

Detection of HAV was made by a PCR technique using the primers described by Tsai et al, 1993. Five microliters of sample were added to 20 µl of the reaction mixture used which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.48 mM of each primer (StabVida) (**HAVC-R** 5'- CTC CAG AAT CAT CTC CAA C - 3' and **HAVC-L** 5'- CAG CAC ATC AGA AAG GTG AG -3'). The primers for HAV capsid amplify a 192-bp.

The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Quantification of rotavirus

The quantification of rotavirus was made by Real time-PCR using the primers described by Villena *et al* (2003). Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0.48 µM of each primer (StabVida) (**VP6-3** 5': GCT TTA AAA CGA AGT CTT CAA C: 3' and **VP6-4** 5': GGT AAA TTA CCA ATT CTT CCA G: 3'). Rotavirus primers positions is found between the position 187 and position 166 of human strain Wa [accession number K02086]).

The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Quantification of enterovirus

The quantification of enterovirus was made by Real time-PCR using the primers described by Beld *et al* (2004). Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0.48 µM of each primer (StabVida) (**Entero 1** 5'- CCC TGA ATG CGG CTA AT -3' and **Entero 2** 5'- ATT GTC ACC ATA AGC AGC CA - 3'). The sequences of the primers used for

amplification are located in the conserved 5' noncoding region of the EV sequence at the nucleotide positions 452 to 468 for Entero-1 and 579 to 597 for Entero-2.
The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Quantification of HAV

The quantification of HAV was made by Real time-PCR using the primers described by Tsai *et al* (1993). Five microliters of sample were added to 20 µl of the reaction mixture consisting of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0.48 µM of each primer (StabVida) (**HAVC-R** 5'- CTC CAG AAT CAT CTC CAA C - 3' and **HAVC-L** 5'- CAG CAC ATC AGA AAG GTG AG -3').
The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Statistical analysis

The statistical analysis of microbiological data was performed using the SPSS21 software. To explain the variation of viral abundance in water, stepwise multiple regression analysis was used. Water temperature, salinity, seston, nutrients and chlorophyll a were used as independent variables.

Enrichment factors

Enrichment factors were calculated dividing the value of viral abundance in SML by the value of viral abundance in UW.

Results

Physical and chemical parameters

The physical and chemical data obtained both in field and in the laboratory, are presented in Table 3.1. Salinity ranged from 20.3 to 35, in the marine zone and from 0.82 to 33 in the brackish water zone, with the higher values in the warm months. The water temperature varied between 10.8 to 21.2°C in the marine zone and between 7.9 to 19.2°C in the brackish water zone, being the values higher in warm months (Table 3.1).

Table 3.1: Physical data in cold (January and February) and warm (June and July) months, in the marine and brackish water zone, in both SML and UW.

Sampling date	Salinity		Water temperature (ºC)	
	MZ	BWZ	MZ	BWZ
January	27.5	0.82	10.8	7.9
February	20.3	1.7	10.9	8.2
June	35	34	20.6	18.3
July	33	30	21.2	19.2

MZ – Marine zone; BWZ – Brackish water zone; SML – Surface microlayer; UW – Under water

Table 3.2: Averaged values and standard deviation of the three samples and for seston and chlorophyll a data in cold (January and February) and warm (June and July) months, in the marine and brackish water zone, in both SML and UW.

				January	February	June	July
Seston (mg L^{-1})		MZ	SML	70.20± 0.33	49.20±0.31	98.40±0.80	66.70±0.84
			UW	44.50±0.14	38.40±0.31	54.44±0.94	48.00±0.60
		BWZ	SML	42.50±0.33	48.89±0.29	98.44±0.16	71.00±0.36
			UW	37.00±0.62	32.50±0.40	67.00±0.33	53.20±0.84
Chlorophyl a (µg L^{-1})		MZ	SML	1.57±0.64	0.96±0.20	2.40±0.01	2.22±0.15
			UW	1.30±0.10	0.50±0.24	1.70±0.11	1.60±0.18
		BWZ	SML	2.31±0.03	2.37±0.06	2.30±0.04	2.38±0.01
			UW	1.00±0.08	1.85±0.09	1.99±0.11	2.16±0.06

MZ – Marine zone; BWZ – Brackish water zone; SML – Surface microlayer; UW – Under water

In Table 3.2 are presented the data obtained for seston and chlorophyll a. Seston values showed a seasonal pattern, with higher concentration in warm months. Seston concentration in the marine zone presented values ranging from 38.40 to 98.40 mg L^{-1} and ranging from 32.50 to 98.44 mg L^{-1} in the brackish water zone. There is not a well-established pattern between the two areas studied, but in both SML has higher seston concentration than UW, with enrichment factors varying from 1.15 to 1.81.

The Chlorophyll a results presented in Table 3.2 show a clear seasonal pattern, with higher values in warmer months, ranging from 0.50 µg L^{-1} to 2.37 µg L^{-1} in cold months

and from 1.60 µg L^{-1} to 2.40 µg L^{-1} in warmer months It was observed a higher concentration of chlorophyll a in the brackish water zone, than in the marine zone. Chlorophyll a values were higher in the SML, than in UW, with an enrichment factor of 1.40 in the marine zone and of 1.34 in the brackish water zone.

Nutrients

The nutrients concentration is showed in Table 3.3. The values were higher in cold months. Amonium concentration ranged from 1.52 to 7.12 µM, nitrates from 2.32 to 17.21 µM, nitrites from 0.15 to 4.90 µM and phosphates from 0.18 to 1.89 µM (Table 3.3).

The nutrients concentration was higher in the SML compared with the UW, with a medium enrichment factor of 1.30 and 1.09 for amonium, of 1.64 and 1.34 for nitrates, of 1.33 and 1.92 for nitrites and of 1.66 and 1.42 for phosphates, in marine and brackish water zone, respectively.

Table 3.3: Nutrients data in cold (January and February) and warm (June and July) months, in the marine and brackish water zone, in both SML and UW.

Sampling Day	Ammonium (µM)				Nitrates/Nitrites (µM)				Phosphate (µM)			
	MZ		BWZ		MZ		BWZ		MZ		BWZ	
	SML	UW	SML	UW	SML	UW	SML	UW	SML	UW	SML	UW
January	2.44	2.22	3.23	3.03	6.92	4.9	17.79	12.05	1.49	1.32	0.23	0.18
February	2.28	2.09	4.83	4.83	14.01	7.08	16.71	10.96	0.76	0.68	1.89	0.46
June	2.62	1.99	7.12	6.32	3.35	2.46	4.88	4.46	0.40	0.37	1.32	0.78
July	2.83	1.52	6.83	6.15	2.86	2.76	4.38	4.06	0.82	0.55	0.61	0.57

MZ – Marine zone; BWZ – Brackish water zone; SML – Surface microlayer; UW – Under water

Enteric viruses

The three viral groups were found in all water samples and Figure 3.2 presents the results of viral abundance for the three groups of enteric virus tested. Rotavirus is the most abundant group (range between 10^8 – 10^{10} copies L^{-1} in cold months and between 10^1 – 10^2 copies L^{-1} in warm months), followed by enterovirus (range between 10^7copies L^{-1} in cold months and 10^1 copies L^{-1} in warm months). HAV was the less abundant group (range between 10^1 – 10^2 copies L^{-1} all year).

All viral groups presented higher concentration in cold months, except for HAV which was almost constant along the year. Viral concentration was higher in the SML, for the three viral groups tested, with enrichment factors varying from 1.01 to 6.37 in the marine zone and from 1.12 to 15.23 in the brackish water zone for rotavirus, from 1.01 to 2.80 in the marine zone and from 1.03 to 5.05 in the brackish water zone for enterovirus and from 1.02 to 2.15 in the marine zone and from 1.17 to 1.44 in the brackish water zone for HAV. There is not clear pattern for the enrichment factors (Table 3.4). Enrichment factors may be underestimated, because SML, as a reservoir for organic matter and other PCR inhibitors, may have greater abundance than it was observed.

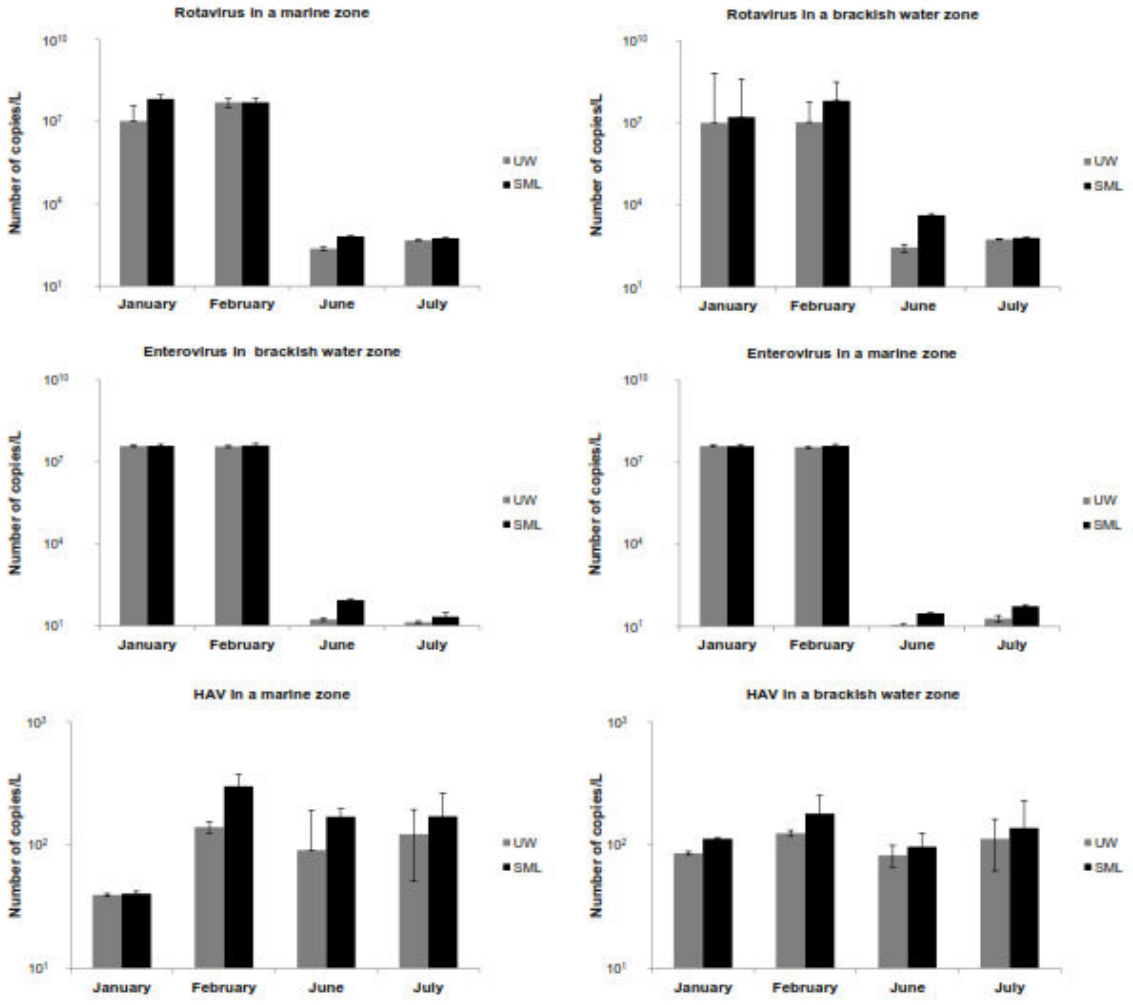

Figure 3.2: Enteric viruses abundance, for the four sampling dates (two in cold months: January and Februry and two in warm months: June and July), in the marine zone (at left) and brackish water zone (at right), for both SML and UW. Values represent the mean of three replicates and error bars represent standard deviation.

Table 3.4: Enrichment factors for rotavirus, enterovirus and HAV in four sampling dates, in the marine and brackish water zones of Ria de Aveiro.

Sampling Date	SML/UW					
	rotavirus		enterovirus		HAV	
	MZ	BWZ	MZ	BWZ	MZ	BWZ
January	6.37	1.63	1.01	1.03	1.02	1.31
February	1.01	6.09	1.10	1.08	2.15	1.44
June	2.77	1.53	2.74	5.05	1.86	1.17
July	1.18	1.12	2.80	1.59	1.40	1.23

MZ – Marine zone; BWZ – Brackish water zone

The results of the stepwise multiple regressions analysis are presented in Table 3.5. None of the independent variables explained the variation of viral abundance for HAV, in both marine and brackish water zones and for rotavirus in the brackish water zone. Water temperature explained almost 100% of viral abndance variation for enterovirus in the brackish water zone and for enterovirus in the marine zone. Nitrites concentration explained almost 100% of viral abundance variation for Rotavirus in the marine water zone.

Table 3.5: Regression equations for viral abundance obtained from stepwise multiple-regression analysis, for marine and brackish water zones. (Dependent variables: enteric virus abundance; independent variables: physical and chemical parameters (salinity, water temperature, DOC, seston, chlorophyll a and nutrients)).

Dependent variable		Independent variables	Regression equation	Adj. R^2
rotavirus	**MZ**	(β= 0.998; p=0.002) Nitrites	Rot= $1.34x10^{'}x - 1.86x10^{6}$	0.993
	BWZ	Not explained by any independent variable		
enterovirus	**MZ**	(β= -0.0998; p= 0.002) Water temperature	$Ent=3.68x10^{6}x+7.71x10^{7}$	0.993
	BWZ	(β= -0.998; p= 0.002) Water temperature	$Ent=-3.27x10^{6}x+6.14x10^{7}$	0.995
HAV	**MZ**	Not explained by any independent variable		
	BWZ	Not explained by any independent variable		

MZ – Marine zone; BWZ – Brackish water zone

Discussion

The sea SML corresponds to the interface between the surface of the water and the air and is generally enriched in organic materials and particles, including microrganisms such as viruses. Consequently SML may play an important role in viral transmission across the air-water interface.However, although much is known about the aquatic specific viruses that at-risk human populations, little is known about their natural reservoirs, namely SML.

The results of this study show that (1) enteric viruses are regularly present in the SML, (2) reaching concentrations significantly higher in this layer than those found in UW, until almost 2 times higher and (3) the abundance of the different enteric viruses is influenced by distinct water characteristics.

The three groups of enteric viruses studied, which are some of the viruses most implicated in human waterborn diseases, were detected in all SML and UW samples, with rotavirus and enteroviruses being the most abundante with similar abundances (range between 10^2 – 10^7 copies L^{-1}). HAV (range between 10^1 – 10^2 copies L^{-1}) is the less abundant. The abundances of rotavirus and enterovirus in the UW are in the range observed for these viruses in other marine systems, 10^1 to 10^7 copies L^{-1} for enteroviruses (Zhang et al, 2010; Chigor and Okoh, 2012; Aslan et al, 2011) and 10^1 to 10^8 copies L^{-1} for Rotavirus (Li et al, 2010; Chigor and Okoh, 2012; Vieira et al, 2012).

Studies on viruses in SML show that they are as abundant as in UW (Parada et al 2005), but up to our knowledge there are no studies about human pathogenic viruses in the SML. The results of this study show that enteric viruses in SML of Ria de Aveiro can reach concentrations higher than those found in UW, until almost 2 times higher. As enteric viruses are frequently transmited by fecal-oral route, they are able to resist to extreme conditions (Pond 2005), such as those observed at SML. This can explain the tendency of enteric viruses present higher concentrations at the SML, where intense solar radiation, high temperature, salinity gradients, toxic organic substances and heavy metals are observed (Liss and Duce 1997). In fact, enteric viruses are environmentally stable, they can live outside of a host for long periods of time (months, or even a year or more) compared to other non-enteric viruses (Rzezutka and Cook, 2004). They are resistant to low pH, in general, are very stable at the pH of most natural waters, pH 5 to 9 (Melnick *et al*. 1978, Duizer *et al*. 2004 Cannon *et al*. 2006, Bosch 2007), survive for several months in seawater at temperatures between 20 and 30ºC (Rzezutka and Cook, 2004), are more resistant to UV radiation from the sun than many other pathogens (Suttle *et al*. 1992, Bosch *et al*. 2006, Suttle *et al*. 2007), salinity does not present a direct effect on viral survival (Lo *et al*, 1976; Fong *et al*, 2005; Bosch *et al*, 2006). Moreover, they are resistant

to detergents and organic solvents frequently present at SML (Liss and Duce 1997). Their association with particulate material prolongs their survival in the marine environment (Rao et al, 1984; Bosch et al, 2005; Fong et al, 2005; Bosch, 2007). In SML, particulate organic matter can be enriched up to 1000 times in the SML compared to UW (Liss and Duce,1997) and viruses associated with small-size particulate material (<3 μm) tend to float in the water column (Grabow, 1996; Bosch et al, 2005; Fong et al, 2005). On the other hand, although these microorganisms may be exposed to SML stress they may also be protected within the organic matrix which could also explain the trend of higher values at SML (Liss and Duce, 1997).

The enterovirus, coxsackievirus, poliovirus and echovirus have been detected in aquatic environment (Fong *et al*. 2005; Chigor and Okoh, 2012; Moresco et al, 2012), being infections in humans reported to peak in summer and early fall, which also coincides with increased water recreational activities and water contact (Fong et al. 2005). However, in this study, the abundance of these viruses was higher in the cold months (January and February) which can be due to the transportation of terrestrial viruses through runoff during the rainning periods. In fact, the levels of nitrites and nitrates, which indicate the presence of freshwater, are around three times higher in the cold months than in the warm months. On the other hand, the lower temperatures observed during the cold months can also explain this pattern of variation. In fact, the variation in enterovirus abundance in both marine and brackish water zones was explained by water temperature, which was two times lower in the cold months. Relatively to rotaviruses, it has been observed that they cause diseases more frequently during the winter months in countries with a temperate climate (Bosch et al, 2005). In this study the rotavirus presents higher abundances in the cold months. Their variation was explained by the levels of nitrites and nitrates, indicating that these viruses can also be transported from the terrestrial neighbourhood. Contrarely, HAV present similar densities in both cold and warm months and its abundance was not explained by any of the studied environmental characteristics. As this virus is not so abundant than the other two, its transportation by runoff could not be so important, and a stable density is maintained in the aquatic environment.

Overall, the studied enteric viruses seem to have mainly terrestrial origin, and can be concentrated on the SML, reaching frequently values around two times higher than those of the UW, which suggests that SML can act as a source of viruses.

Acknowledgements

The authors would like to thank the University of Aveiro and Centre for Environmental and Marine Studies (CESAM) for funding the study. Financial support to C. Prata was provided by the Portuguese Foundation for Science and Technology (FCT) in the form of a PhD grant (SFRH/BD/33390/2008), São José Nascimento of University of Porto and Albert Bosh of University of Barcelona, for providing viral stocks used in this work as positive controls.

References

- Albinana-Gimenez N, Clemente-Casares P, Bofill-Mas S, Hundesa A, Ribas F, Girones R, 2006, Distribution of human polyomaviruses, Adenoviruses and hepatitis E virus in the environmental and drinking-water treatment plant, Environmental Science and Technology, 40, 7416–7422.
- Allard A, Albisson B, Wadell G, 2001, Rapid Typing of Human Adenoviruses by a General PCR combined with Restriction Endonuclease Analysis, Journal of Clinical Microbiology, 39 (2) 498–505
- Aslan A, Xagoraraki I, Simmons F J, Rose J B, Dorevitch S, 2011, Occurrence of adenovirus and other enteric viruses in limited-contact freshwater recreational areas and bathing waters, Journal of Applied Microbiology, 111, 1250–1261.
- Beld M, Minnaar R, Weel J, Sol C, Damen M, vanBreda A, Boom R, 2004, Highly Sensitive Assay for Detection of Enterovirus in Clinical Specimens by Reverse Transcription-PCR with an Armored RNA Internal Control, Journal of Clinical Microbiology, 42 (7) 3059–3064
- Bosch A, Abad F X, Pintó R M, 2005, Human Pathogenic Viruses in the Marine Environment, Oceans and Health: Pathogens in the Marine Environment, 109-131
- Bosch A, Abad F X, Pintó R M, 2006, Survival and transport of enteric viruses in the environment, In “Viruses in Food”, S. M. Goyal (Ed.), Food Microbiology and Food Safety Series, Springer, New York, pp 151-187.
- Bosch A, 2007, Viruses Ocurrence and Survival in the Environmental waters, In “Human Viruses in water”, First Edition, Elsevier, pp 91-108.
- Cannon J L, Park G W, Osborne J, Jaykus L A and Vinje J, 2006, Surrogates for the Study of Norovirus Stability and Inactivation in the Environment: A Comparison of Murine Norovirus and Feline Calicivirus, Journal of Food Protection, 69(11) 2761–2765.
- Cunliffe M, Upstill-Goddard R C, Murrell J C, 2011, Microbiology of aquatic surface microlayers, FEMS Microbiological Reviews, 1–14.

- Dias J M, Lopes J F, Dekeyser I, 2000, Tidal propagation in Ria de Aveiro lagoon, Portugal. Physical and Chemistry Earth;25, 369-74
- Duizer E, Bijkerk P, Rockx B, Groot A, Twisk F, Koopmans M, 2004, Inactivation of Caliciviruses, Applied and Environmental Microbiology, 70(8).4538–4543.
- Fong T-T, Lipp E K, 2005, Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools, Microbiology and Molecular Biology reviews, 69 (2) 357-371.
- Grabow W, 1996, Waterborne diseases: Update on water quality assessment and control, Water SA, 22(2), 193-202.
- Griffin D W, Gibson III C J, Lipp E K, Riley K, Paul III J H, Rose J B, 2003, Detection of Viral Pathogens by Reverse Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys, Applied and Environmental Microbiology, 65(9): 4118-4125.
- Hamza I A, Jurzik L, Stang A, Sure K, Uberla K, Wilhelm M, 2009, Detection of human viruses in rivers of a densely-populated area in Germany using a virus adsorption method optimized for PCR analyses, Water Research, 43, 2657–2668.
- Hardy J T, 1982, The sea-surface microlayer – biology, chemistry and anthropogenic enrichment, Prog Oceanography, 11, 307–328.
- Harvey G W, Burzell L A, 1972, A simple microlayer method for small samples. Limnology and Oceanogrphy, 11, 156–157.
- Koroleff F, 1970, Direct determination of ammonia in natural water as indophenol blue. *ICES, Pap. C.M.* 1969/C:9, revised 1970, 19-22.
- Lee S H, Kim S J, 2002, Detection of infectious enteroviruses and Adenoviruses in tap water in urban areas in Korea, Water Research, 36(1), 248–256.
- Li D, Gu Z, Yang W, He M, Hu X-H, Shi H-C, 2010, An integrated cell culture and reverse transcription quantitative PCR assay for detection of infectious rotaviruses in environmental waters, Journal of Microbiological Methods, 82, 59–63.
- Liss P S, Duce R A, 1997, The Sea Surface and Global Change, Cambridge University Press, 519 pp.
- Lo S, Gilbert J, Hetrick F, 1976, Stability of Human Enteroviruses in Estuarine and Marine Waters, Applied and Environmental Microbiology, 32 (2) 245-249.
- Lorezen C J, 1967, Determination of chlorophyll and phaeopigments: spectrophotometric equations, Limnology and Oceanography, 12 (2):343– 6.

- Lugoli F, Leopizzi M I, De Donno A, 2009, Viral Indicators of Hygienic-Sanitary Quality: Detection of Somatic Coliphages in the Southern Adriatic Sea, Water Qual Expo Health, 1, 171-177.
- Melnick J L, Gerba C P, Wallis C, 1978, Viruses in water, Bulletin of the World Health Organization, 56 (4): 499-508.
- Moresco V, Viancelli A, Nascimento M A, Souza D S M, Ramos A P D, Garcia L A T, Simões C M O, Barardi C R M, 2012, Microbiological and physicochemical analysis of the coastal waters of southern Brazil, Marine Pollution Bulletin, 64 (1) 40-48.
- Myrmel M, Berg E M M, Grinde B, Rimstad E, 2006, Enteric viruses in inlet and outlet samples from sewage treatment plants, Journal of Water Health, 197–209.
- Noble R T, Fuhrman J A, 1998, Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria, Aquatic Microbial Ecology, 14, 113–118.
- Parada V S, Sintes E, Weinbauer M, Herndl G, 2005, Dynamics in the Sea Surface Microlayer in an anticyclonic eddy of the Northwestern Mediterranean Sea, Journal of Virioplankton.
- Parsons T R, Maita Y, Lalli C M, 1989, A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford, pp. 172.
- Pianetti A, Baffone W, Citterio B, Caçarola A, Bruscoline F, Salvaggio L, 2000, Presence of enteroviruses and reoviruses in the waters of the Italian coast of the Adriatic Sea, Epidemiology and Infections, 125, 455-462.
- Pond K, 2005, Water recreation and disease: plausibility of associated infections: Acute Effects, Sequelae and Mortality, World Health Organization.
- Prata C, Ribeiro A, Cunha A , Gomes N C M, Almeida A, 2011, Ultracentrifugation as a direct method to concentrate viruses in environmental waters: virus-like particles enumeration as a new approach to determine the efficiency of recovery, Journal of Microbiological Methods, 14 (1), 64-70.
- Rao V C, Seidel KN, Goyal S M, Metcalf T C, Melnick J L, 1984, Isolation of enteroviruses from water, suspended solids and sediments from Galveston bay; survival of poliovirus and rotavirus adsorbed to sediments, Applied and Environmental Microbiology, 48 (2), 404–409.
- Rodier J, 1996, L'analyse de l'eau: eaux naturelles, eaux résiduaires, eau de mer, 8^{e} edn. Dunod, Paris.

- Rodriguez-Diaz J, Querales L, Caraballo L, Vizzi E, Liprandi F, Takiff H, Betancourt W Q, 2009, Detection and characterization of waterborne gastroenteritis viruses in urban sewage and sewagepolluted river waters in Caracas, Venezuela, Applied and Environmental Microbiology, 75(2), 387–394.
- Rzeżutka A, Cook N, 2004, Survival of human enteric viruses in the environment and food, FEMS Microbiology Reviews, 28, 441–453.
- Sedmak G, Bina D, Macdonald J, Couillard L, 2005, Nine-year study of the occurrence of culturable viruses in source water for two drinking water treatment plants and the influent and effluent of a wastewater-treatment plant in Milwakee, Wisconsin, Applied and Environmental Microbiology, 71, 1042–1050.
- Silva J J, 1994, Circulação de água na Ria de Aveiro: Contribuição para o estudo da qualidade da água, PhD Thesis, Universidade de Aveiro.
- Suttle C A, 2005, Viruses in the sea, Nature, 437, 356-361.
- Suttle C A, 2007, Marine viruses — major players in the global ecosystem, Nature, 5, 801-812.
- Tsai Y L, Sobsey M D, Sangermano L R, Palmer C J, 1993, Simple Method of Concentrating Enteroviruses and Hepatitis A Virus from Sewage and Ocean Water for Rapid Detection by Reverse Transcriptase-Polymerase Chain Reaction, Applied and Environmental Microbiology, 59 (10), 3488-3491.
- Vennema H, Bruin E, Koopmans M, 2002, Rational optimization of generic primers for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction, Journal of Clinical Virology, 25, 233-235.
- Villena C, El-Senousy W M, Abad F X, Pinto R M, Bosch A, 2003, Group A rotavirus in sewage samples from Barcelona and Cairo: emergence of unusual genotypes, Applied and Environmental Microbiology, 69 (7) 3919–3923.
- Weinbauer M G, Christaki U, Nedoma J, Simek Karel, 2004, Comparing the effects of resource enrichment and grazing on viral production in a meso-eutrophic reservoir, Aquatic Microbial Ecology, 31 (2) 137-144.
- Wyn-Jones A P, Sellwood J, 2001, Enteric viruses in the aquatic environment. A Review, Journal of Applied Microbiology 91, 945-962.
- Yentsch C S, Menzel D W, 1963, A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence, Deep-Sea Research 10, 221–231.
- Zhang M, Zhao H, Yang J, Jiang S, Cai B, 2010, Detection and quantification of enteroviruses in coastal seawaters from Bohai Bay, Tianjin, China, Journal of Environmental Sciences, 22 (1) 150–154.

Chapter 4 - Marine aerosols as a source of health risk enteric virus

Marine aerosols as a source of health risk enteric virus

[1]Catarina Prata, [1]Ângela cunha, [1]Newton Gomes, [2]Teresa Nunes, [1]Adelaide Almeida*

[1]Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2]Department of Environment and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

*Corresponding author: E-mail: aalmeida@ua.pt, Tel.: +351234370350, Fax: +351234372587

ABSTRACT

Viruses are nowadays considered to be emerging pathogens in the marine environment. Due to their small infectious dose, it is of extreme importance to evaluate if they can be transferred from water to air through aerosol formation. The aim of this study was to evaluate the role of aerosols in the transference of health relevant viruses from marine waters to air.

It was developed a method to create and recover aerosols, using Virus-like particles (VLP) to access the efficiency of the method. It was used a polycarbonate membrane (0.2 µm pore) to remove virus from the air used do generate aerosols, with an efficiency of almost 80%.

The abundance of three enteric virus groups (enterovirus, rotavirus and HAV) was quantified in underwater (UW), surface microlayer (SML) and in aerosols simulated by bubbling in marine and brackish waters zones of Ria de Aveiro (Portugal). The three enteric viruses abundances were higher in SML relatively to the UW and all of them were detected in aerosols, although at concentrations significantly lower than those found in the SML. The data indicate that SML is an important source of viruses entering the atmosphere trough aerosols formation.

INTRODUTION

Some health relevant enteric viral groups are nowadays considered to be emerging marine waterborne pathogens (La Rosa et al, 2012; Woods, 2013), since they are able to adapt not only by mutation but also through recombination and reassortment and can thus become able to infect new hosts and to adapt to new environments (La Rosa et al, 2012).

Enteric viruses can be transmitted to humans through marine waters used for recreational purpose or from waters used to shellfish growing, but also through aerosols transmition from the sea surface microlayer (SML). The sea SML is generally defined as the uppermost millimeter of the ocean (Liss and Duce 1997). Consequently, the primary interest in the

structure and function of the SML is based on its crucial role in exchange processes of gases and matter across the air-water interface (Agougué et al, 2004; Cunliffe et al, 2011; Donaldson and George, 2011). Marine aerosols are formed primarily by the eruption of rising bubbles through the SML (Aller et al, 2005) and constitute the largest fraction of atmospheric aerosol loading over the remote open oceans, contributing to regional air quality (Brooks et al, 2011) and through sea-to-air transfer of bacteria and viruses (Mathias-Maser and Jaenicke, 1994; Posfai et al, 2003; Brooks et al, 2011), transporting microbes for long distances (Moorthy et al, 1998; Klassen and Roberge, 1999; Chow et al, 2000; Gutafsson and Franzen, 2000; Grammatika and Zimmerman, 2001). Aerosols may remain suspended in the atmosphere for weeks (Dueñas et al, 2004) and it is known that many microorganisms, as well as viruses, can remain infectious outside their hosts for prolonged periods of time (Verrault et al, 2008). Despite the lack of information for viral survival in aerosols, there are evidences that at least some marine bacteria remain viable after overland transport in aerosols (200 m inland, collected at 30 m above sea level) (Marks et al, 2001), despite exposure to ultraviolet radiation, changes in temperature and salinity and other immediate stresses. Since some health relevant viruses are waterborne emerging pathogens in the marine environment and their infectious doses are small, it is of extreme importance to understand if it is possible that these pathogens pass from water to air, and if they can be disperse inside the aerosols (Bosch et al, 2011). Studies on the aerobiology of infectious diseases have been rather limited (Roy and Milton, 2004), mainly due to the difficulty in collecting and analyzing airborne biological contaminants, which is an even greater problem for viruses (Verrault et al, 2008; Danovaro et al, 2011).

The aim of this study was to evaluate the role of aerosols in the transference of enteric virus from marine waters to air. For this, the abundance of three enteric viruses (enterovirus, rotavirus and HAV) was quantified in UW, SML and in aerosols, simulated by bubbling water, in two different locations (marine zone and brackish water zone) of Ria de Aveiro (Portugal). The abundance of virus-like particles (VLP) was also determined in order to develop the aerosol collection protocol, to decide the collection medium, the sampling period and the membrane to filtrate the air to be used to form the aerosols. The VLP was also determined in the UW, SML and in the simulated aerosols.

Material and Methods

Development of aerosols collection protocol in laboratory

Aerosols collection medium

Destilated miliQ water was tested as an aerosols collection medium (Yu et al, 2010), The air was collected in 10 mL of distelated water, by impingement, for 5 hours with a flow rate of 2 L min^{-1}. The collection medium was tested using the bundance of VLP. Three samples were prepared.

Determination of membrane efficiency to remove viruses from the bubbling air

A 0.2 µm pore polycarbonate membrane was tested to remove the viruses from the bubbling air used to produce the aerosols. Ten milliliters of collection medium were placed in a glass chamber and the filtered air was used to bubble the water, at a controlled flow rate of 2 L/min, for 5 hours. The same procedure was done in the absence of the membrane (control). Three samples were processed with polycarbonate membranes and three without membrane. The polycarbonate membrane efficiency was determined using the abundance of VLP for all samples with and without membrane.

Sampling time for aerosols formation in laboratory

The aerosols formation was tested using 10 mL of water sample collected in a brackish zone of Ria de Aveiro (Station I6, located in Canal de Ílhavo, see information below), in three diferrent dates in January 2012, by submerging a glass bottle 15 cm in the water column. Sampling times tested were 30 minutes and 1 hour of air filtration, in order to determine the minimal time needed to form aerosols containing an enough amount of VLP. The aerosols sampler used was an all glass impinge with the nozzle positioned 10 mm above the bottom and suction was applied to the small side arm. Ten milliliters of water sample were placed in a glass chamber of the impinge and 10 mL of miliQ water were used to recover the aerosols formed by bubbling filtered air into the water sample, at a controlled flow rate of 2 L min^{-1}.
For each sampling time, three samples and a negative control of the air, consisting in filtered air bubbling directly into the miliQ water, were used. The VLP was determined in the samples and in the control by epifluorescence microscopy.

Concentration of aerosols samples

After aerosols formation and recovery, samples were concentrated, using the ultracentrifugation method, as described by Prata et al (2012). Briefly, samples were centrifuged (Beckman Optima™, LE-80K Ultracentrifuge, rotor 50.2 Ti) at 100,000 x g for 1

hour at 20°C and the pellet was ressuspended in 200 µl of 1x PBS, which was used to count the VLP by epifluorescence microscopy.

Determination of VLP in aerosols samples

VLP abundance was determined using a modified epifluorescence method described by Noble and Fhurman (1998). Water samples were filtered by a 0.2 µm polycarbonate membrane and then by a 0.02 µm Al2O3 Anodisc, which were than stained in the dark for 10 minutes with SYBR gold dye (0.25%). VLP were enumerated in an epifluorescence up-right microscope Imager.Z1 (Zeiss) with 38 HE GFP filter.

For each sample two slides were made and a minimum of 200 particles per slide were counted. Three independent assays were done for each sampling date.

Field work

Study site

Ria de Aveiro is a branched estuarine ecosystem, located in the northwest coast of Portugal, connected to the Atlantic by a narrow opening and separate from the sea by a sand bar. The Ria covers an area of 66 to 83 km^2 at low and high tide, respectively (Silva, 1994; Dias et al, 2000).

Three samples of SML, UW and aerosols were collected in two locations: marine zone (Station N1) and brackish water zone (Station I6) in Canal de Ílhavo (Figure 4.1), both in Ria de Aveiro, three times, between July and August of 2012.

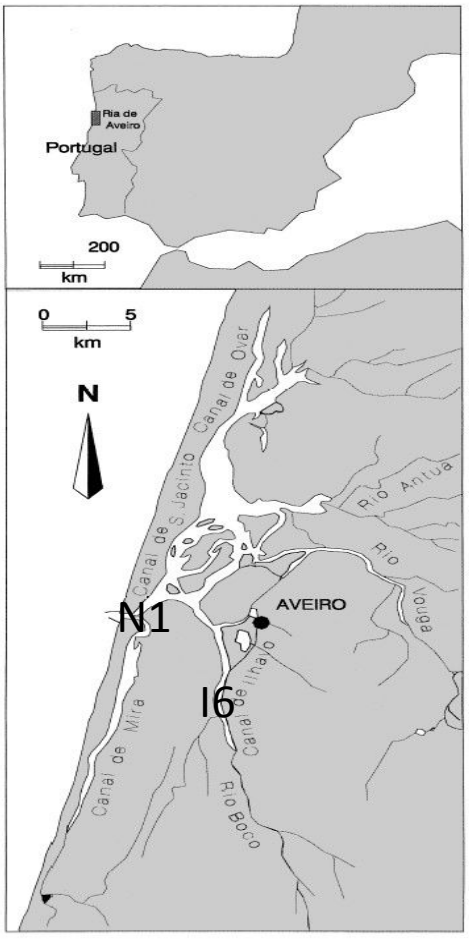

Figure 4.1: The estuarine system Ria de Aveiro with an indication of the sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ilhavo, represents the brackish water zone.

In situ generation and collection of aerosols

Aerosols were generated *in situ* using a small floating device. According to the results of obatained in the laboratory experiments, a 0.2 µm pore polycarbonate membrane was used to remove the viruses from the bubbling air used to produce the aerosols and the bubbling was done at 2 L min^{-1} for 1 hour. The aerosols formed were collected in 10 mL of miliQ water, with two replicates per sample in each date. An air control sample was also included for each sample. Three independent samples were collected for each sampling date.

SML and UW sampling

Samples of 0.5 L of SML were collect with plexiglass and glass plates (0.25 m wide, 0.35 m long and 4 mm thick). Prior to sample collection, the plates were rinsed with ethanol and sterile distilled water. The plates were introduced vertically through the SML and withdrawn in the same position. Excess water was allowed to drain for about 5 s. Approximately 5 mL of water were collected each time the plates were introduced into the SML. The water adhering to the plate was subsequently removed from both sides of the plate with a wiper blade system (Harvey and Burzell 1972). The estimated thickness of the collected SML, determined from the volume of collected sample and the area of both sides of the plate, was approximately 60 µm, which is in the range reported by Harvey and Burzell (1972) for glass plate samplers. Samples of 0.5 L of subsurface water were collected by submerging a glass bottle 15 cm in the water column. Three independent samples were collected for each sampling date. Samples were maintained at 4 °C until analysis.

Concentration of water and aerosols samples

Aerosols samples were concentrated, using the ultracentrifugation method described above in Concentration of aerosols samples.

Abundance of VLP in water and aerosols samples

VLP abundance was determined using the modified epifluorescence method described above in the development of aerosols collection protocol in laboratory.

Nucleic acids extraction

Nucleic acids were extracted from concentrated water and aerosol samples, using the geneMAG-RNA/DNA kit, a magnetic RNA/DNA purification kit (Chemicell ™), according to the instructions of the manufacturer. cDNA synthesis was made using a NZY First-Strand cDNA Synthesis Kit (Nzytech), using the forward primers described below in the PCR technique. The nucleic acids were stored at -80°C until analysis.

Enteric virus detection and quantification

All viral groups were detected using a TPersonal thermocycler (Biometra) for PCR technique. The amplification products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and detected with UV transilumination.

Quantification was made using a stepone plus 46 weel thermocycler (Applied Biosystems) and standards were obtained from a serial dilution of a suspension of each viral group, with a known initial number of copies. Negative controls were made, by using sterilized miliQ-water, instead of nucleic acids.

Detection of rotavirus

Detection of rotavirus was made by a PCR technique using the primers described by Villena et al, 2003. Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.48 mM of each primer (StabVida) (**VP6-3** 5': GCT TTA AAA CGA AGT CTT CAA C: 3' and **VP6-4** 5': GGT AAA TTA CCA ATT CTT CCA G: 3'). Rotavirus primers position is found between the position 187 and position 166 of human strain Wa [accession number K02086]), creating a fragment of 186-bp.

The amplification was carried out for 40 cycles of 94ºC for 10 s, 50ºC for 30 s and 72ºC for 20 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Detection of enterovirus

Detection of enterovirus was made by a PCR technique using the primers described by by Beld et al, 2004. Five microliters of sample were added to the reaction mixture which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.48 mM of each primer (StabVida) (**Entero 1** 5'- CCC TGA ATG CGG CTA AT -3' and **Entero 2** 5'- ATT GTC ACC ATA AGC AGC CA - 3'). Enterovirus primers sequences used for amplification are located in the conserved 5' noncoding region of the EV sequence. Nucleotide positions 452 to 468 for Entero-1 and 579 to 597 for Entero-2, creating a fragment of 172-bp.

The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Detection of HAV

Detection of HAV was made by a PCR technique using the primers described by Tsai et al, 1993. Five microliters of sample were added to the reaction mixture which consisted of 2 mM Buffer taq, 3 mM MgCl2, 0.08 mM of each dNTP, 0.1 U/µL of Taq polymerase (Fermentas) and 0.48 mM of each primer (StabVida) (**HAVC-R** 5'- CTC CAG AAT CAT CTC CAA C - 3' and **HAVC-L** 5'- CAG CAC ATC AGA AAG GTG AG -3'). The primers for HAV capsid amplify a 192-bp.

The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Quantification of Rotavirus

Quantification of Rotavirus was made by Real time-PCR using the primers described by Villena *et al* (2003). Five microliters of sample were added to the reaction mixture used which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0.48 µM of each primer (StabVida) (**VP6-3** 5': GCT TTA AAA CGA AGT CTT CAA C: 3' and **VP6-4** 5': GGT AAA TTA CCA ATT CTT CCA G: 3'). Rotavirus primers positions is found between the position 187 and position 166 of human strain Wa [accession number K02086]), creating a product of 186-bp.

The amplification was carried out for 40 cycles of 94ºC for 10 s, 50ºC for 30 s and 72ºC for 20 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Quantification of Enterovirus

Quantification of enterovirus was made by Real time-PCR using the primers described by Beld *et al* (2004). Five microliters of sample were added to the reaction mixture which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0.48 µM of each primer (StabVida) (**Entero 1** 5'- CCC TGA ATG CGG CTA AT -3' and **Entero 2** 5'- ATT GTC ACC ATA AGC AGC CA - 3'). The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Quantification of HAV

Quantification of HAV was made by Real time-PCR using the primers described by Tsai *et al* (1993). Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0.48 µM of each primer (StabVida) (**HAVC-R** 5'- CTC CAG AAT CAT CTC CAA C - 3' and **HAVC-L** 5'- CAG CAC ATC AGA AAG GTG AG -3'). The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Statistical analysis

SPSS21 was used for statistical data analysis. The significance of differences in VLP between filtered and non-filtered samples and differences in the number of virus like particles among aerosols samples, SML and UW was assessed using one-way ANOVA. Data were tested for normal distribution (assessed by the Kolmogorov–Smirnov test) and homogeneity of variances (assessed by Levene's test). A value of $p < 0.05$ was considered significant at a confidence level of 95%.

Enrichment factors

Enrichment factors were calculated dividing the value of viral abundance in SML by the viral abundance value of UW and aerosols.

Results

Development of aerosols collection protocol in laboratory

Sampling time for aerosols formation in laboratory

Thirty minutes of aerosols formation was not sufficient to have enough amount of viruses for subsequent analysis (only 2-3 VLP/ microscopy field), but 1 hour of aerosols formation shown to be sufficient to have enough amount of viruses (100-200 VLP/ microscopy field). This sampling time was selected to be used in the field work.

Filter efficiency to remove viruses from the bubbling air

The polycarbonate membrane with 0.2 µm pore was efficient in removing viruses from the air. An efficiency of almost 80% was observed in three different sampling dates (Table 4.1).

Significant differences in the number of VLP determined between filtered and not-filtered samples ($p=0.0003 < 0.05$) were observed.

Values presented in Table 4.1 represent the mean value of VLP of three replicates, for each sampling date, to determine the efficiency of the polycarbonate membrane in removing virus from the air used to form aerosols.

Table 4.3: Filter efficiency in removing viruses from the air in three sampling dates.

Sampling	VLP/ml with filter	VLP/ml without filter	Filter Efficiency (%)
Day 1	$2.97 \times 10^8 \pm 2.47 \times 10^7$	$1.42 \times 10^9 \pm 0.02 \times 10^9$	79.12
Day 2	$3.39 \times 10^8 \pm 1.48 \times 10^7$	$1.61 \times 10^9 \pm 0.19 \times 10^9$	78.91
Day 3	$3.91 \times 10^8 \pm 5.92 \times 10^7$	$1.57 \times 10^9 \pm 0.17 \times 10^9$	78.11

The values represent the average and standard deviation of the three samples.

Field Work

Abundance of VLP

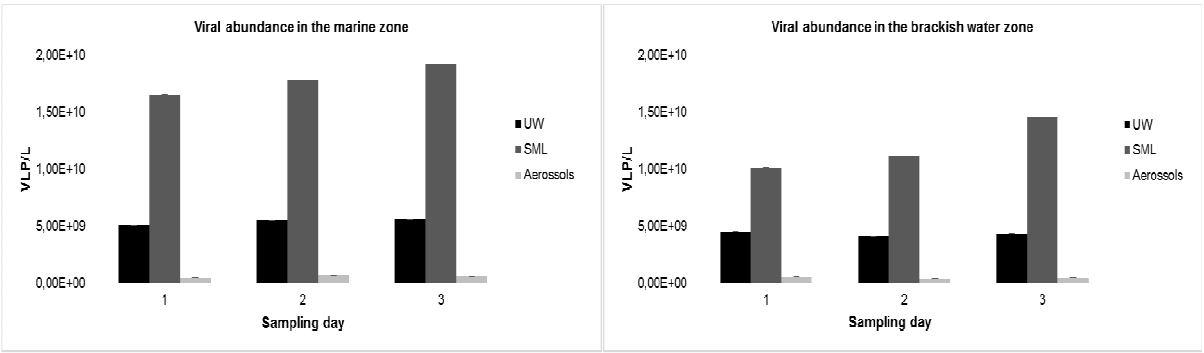

Figure 4.2: Abundance of VLP for UW, SML and Aerosols, in a marine (left) and a brackish water zone (right) of Ria de Aveiro, for three sampling days. Values represent the mean of three independent experiments; error bars indicate the standard deviation.

Figure 4.2 representes the abundance of VLP, for UW, SML and aerosols, in a marine and a brackish water zone. Both areas studied show the same pattern, VLP are more concentrated in SML, followed by UW and, aerosols. Viral abundance is similar in both zones and ranges from about 10^9 to 10^{10} in SML and UW and is about 10^8 in aerosols.

Statistical analysis shows significant differences between VLP on aerosols, SML and UW samples for the marine zone ($p < 0.05$) and for the brackish water zone ($p < 0.05$).

The SML presents greater abundance, relatively to UW and Aerosols and the enrichment is higher in the marine zone, when compared to the brackish water zone. The enrichment factors obtained for the marine zone were of 2.78±0.57 and 26.14±6.21, for SML regardless

to UW and aerosols, respectively. In the brackish water zone, the enrichment factors obtained were of 3.32±0.12 and 33.22±4.08, for the SML regardless to UW and aerosols, respectively.

Abundance of enteric viruses

The pattern of variation of enteric viruses among UW, SML and aerosols samples in the marine and brackish water zones was similar (Figure 4.3). The abundance of the three viruses was, in general, higher in SML samples, followed by UW samples and aerosol samples. The exception was found for rotavirus in the BWZ and for enterovirus in the MZ, that present similar abundance beteeen the SML and the UW.

In general, rotavirus was the most abundant virus in UW, SML and aerosols and enterovirus was, in general, the lowest (Figure 4.3).

The ratio between SML and UW values (enrichment factors) varied between 1.47 and 5.70 in the marine zone with an average of 2.80 ± 4.40 (Table 4.2). In the brackish water zone, the enrichment factors ranged from 1.06 to 8.25, with an average of 2.97 ± 1.36.

The ratios between SML and aerosol values varied from 5.36 to 37.22 in the marine zone, with an average of 21.57 ± 15.45 and from 4.54 to 31.97 in the brackish water zone, with an average of 14.63 ± 9.59. Enrichment factors may be underestimated, because SML, as a reservoir for organic matter and other PCR inhibitors, may have greater abundance than it was observed.

Table 4.2: Enrichment factores for rotavirus, enterovirus and HAV in three sampling dates, in a marine and in a brackish water zone of Ria de Aveiro.

Sampling Date	SML/UW						SML/Aerosols					
	Rotavirus		Enterovirus		HAV		Rotavirus		Enterovirus		HAV	
	MZ	BWZ	MZ	BWZ	MZ	BWZ	MZ	BWZ	MZ	BWZ	MZ	BWZ
1	2.72	1.09	1.49	1.14	3.81	3.34	5.58	7.56	32.92	31.97	6.01	4.54
2	2.89	1.17	1.75	1.10	5.70	6.96	36.17	12.29	36.77	26.03	5.36	6.74
3	3.03	1.06	1.47	1.09	3.82	8.25	29.14	14.63	37.22	20.58	5.00	7.55

MZ – Marine zone

BWZ – Brackish water zone

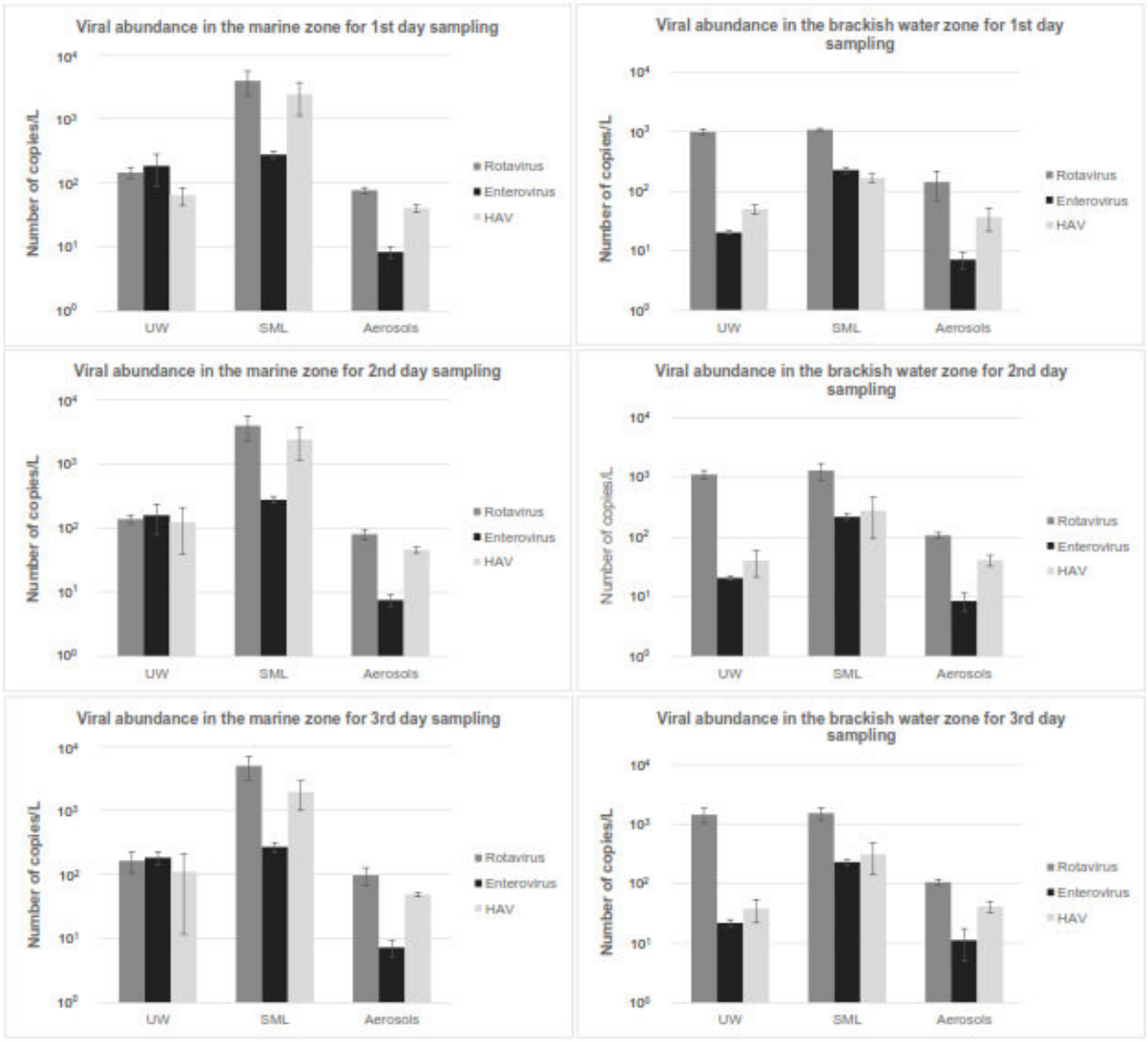

Figure 4.3: Abundance of enteric viruses for UW, SML and aerosol samples in the marine (left) and brackish water (right) zones of Ria de Aveiro, for the three sampling dates. Values represent the mean of three samples and error bars represent the standard deviation.

Discussion

Few studies have been done to estimate the concentration of different viruses in the air as well as to identify their source strength (Després et al, 2011), mainly due to difficulties in collecting and analyzing airborne viruses (Verrault et al, 2008). However, Aller et al (2005) presented results supporting that SML acts as a major source of viruses to the atmosphere, being aerosolization an important mechanism of viral dispersion to long distances. The results of this study also support the idea that SML acts as a reservoir for enteric viruses and that aerosols are an important vehicle for virus diffusion. In the three sampling dates the three enteric viruses groups tested were higher in SML relatively the UW and all of them

were detected in aerosols although at concentrations significantly lower than those found in the SML. Enteroviruses, frequently, presented a much lower concentration in aerosols than that detected in the SML. This suggests that this group of virus can present a lower survival in the air than the other two groups. As the rotavirus is a dsRNA virus its survival is higher than that of enterovirus, a ssRNA virus, since viruses with dsRNA may use their undamaged RNA strand as a template to host repair the UV light damage (Suttle *et al*. 1992, Gerba *et al.* 2002, Bosch *et al*. 2006, Bosch 2007, Bosch *et al*. 2008). However, the HAV is also a ssRNA virus, but this behavior was not observed for this group of virus. As the genome size and morphology of these both viruses is similar, both belong to the Picornaviridae family (Griffin *et al.* 2003, Mahy *et al*. 2009), more studies are needed to explain the different behavior of these two groups in the air. It will be important to evaluate if enterovirus and HAV viruses have, for instance, nucleic acids repair systems.

The results of this study indicate that the SML of Ria de Aveiro is an important source of viruses that are transported to the atmosphere by aerosol formation, but it is not possible to compare the importance of this source of enteric viruses with that of other systems because there are no available data.

As the unavailability of data about the concentration of viruses in the air and about their source is mainly due to difficulties in collecting and analyzing airborne viruses, the development of a simple, fast and unexpensive approach is essential. The method developed in this study gathers these requeriments. Moreover, the enumeration of the total VLP by epifluorescence instead the enumeration of a specific virus indicates that this approach is an appropriate choice to collect and quantify any type of viruses present in the atmosphere.

Acknowledgements

The authors would like to thank the University of Aveiro and Centre for Environmental and Marine Studies (CESAM) for funding the study. Financial support to C. Prata was provided by the Portuguese Foundation for Science and Technology (FCT) in the form of a PhD grant (SFRH/BD/33390/2008), São José Nascimento of University of Porto and Albert Bosh of University of Barcelona, for providing viral stocks used in this work as positive controls.

References

- Agogué H, Casamayor E O, Joux F, Obernosterer I, Dupuy C, Lantoine F, Catala P, Weinbauer M G, Reinthaler T, Herndl G J, Lebaron P, 2004, Comparison of samplers for the biological characterization of the sea surface microlayer, Limnology and Oceanography Methods, 2, 213–225.
- Allard A, Albisson B, Wadell G, 2001, Rapid Typing of Human Adenoviruses by a General PCR combined with Restriction Endonuclease Analysis, Journal of Clinical Microbiology, 39 (2) 498–505.
- Aller J Y, Kuznetsova M R, Jahns C J, Kemp P F, 2005, The sea surface microlayer as a source of viral and bacterial enrichment in marine aerosols, Aerosol Science, 36, 801–812.
- Beld M, Minnaar R, Weel J, Sol C, Damen M, vanBreda A, Boom R, 2004, Highly Sensitive Assay for Detection of Enterovirus in Clinical Specimens by Reverse Transcription-PCR with an Armored RNA Internal Control, Journal of Clinical Microbiology, 42 (7) 3059–3064
- Bosch A, Abad F X, Pintó R M, 2006, Survival and transport of enteric viruses in the environment, In “Viruses in Food”, S. M. Goyal (Ed.), Food Microbiology and Food Safety Series, Springer, New York, pp 151-187.
- Bosch A, 2007, Viruses Ocurrence and Survival in the Environmental waters, In “Human Viruses in water”, First Edition, Elsevier, pp 91-108.
- Bosch A, Guix S, Sano D, Pinto R M, 2008, New tools for the study and direct surveillance of viral pathogens in water, Current Opinion in Biotechnology, 19, 295–301.
- Bosch A, Sánchez G, Abbaszadegan M, Carducci A, Guix S, Le Guyader F S, Netshikweta R, Pintó R M, van der Poel W H M, Rutjes S, Sano D, Taylor M B, van Zyl W B, Rodríguez-Lázaro D, Kovač K, Sellwood J, 2011, Analytical Methods for Virus Detection in Water and Food, Food Analytical Methods,4 (1), 4-12.
- Brooks I M, Andreas E L, McFIiggans G, Anguelova M D, O’Dowd C, 2011, Primary Marine Aerosol Fluxes: Progress and Priorities, American Meteorological Society, 489-491.
- Chow J C, Watson J G, Green M C, Lowenthal D H, Bates B, Oslund W, Torres G, 2000, Cross-border transport and spatial variability of suspended particles in Mexicali and California's Imperial Valley, Atmospheric Environment, 34 (11) 1833–1843.
- Cunliffe M, Upstill-Goddard R C, Murrell J C, 2011, Microbiology of aquatic surface microlayers, FEMS Microbiological Reviews, 1–14.

- Danovaro R, Corinaldesi C, Dell'Anno A, Fuhrman J. A, Middelburg J J, Noble R T, Suttle C A, 2011, Marineviruses and global climate change, FEMS Microbiology Reviews, 35, 993–1034.
- Després V R, Huffman J A, Burrows S M, Hoose C, Safatov A S, Buyak G, Fröhlich-Nowoisky J, Elbert W, Andreae M, Pöschil U, Jaenicke R, Tellus B, 2012, Primary biological aerosol particles in the atmosphere: a review, Chemical and Physical Meteorology, 64, 15598.
- Dias J M, Lopes J F, Dekeyser I, 2000, Tidal propagation in Ria de Aveiro lagoon, Portugal. Physical and Chemistry Earth;25, 369-74
- Donaldson D J, George C, 2011, Introduction to the Focus Issue on Marine Boundary Layer: Ocean Atmosphere Interactions Processes, Environmental Science Technology, 46, 10383−10384.
- Dueñas C, Fernandez M C, Carretero J, Liger E, Cañete S, 2004, Long-term variation ofthe concentrations of long-lived Rn descendants and cosmogenic ^{7}Be and determination ofthe MRT ofaerosols, Atmospheric Environment, 38, 1291–1301.
- Gerba C P, Gramos D M, Nwachuku N, 2002, Comparative Inactivation of Enteroviruses and Adenovirus 2 by UV Light, Applied and Environmental Microbiology, 68 (10) 5167–5169.
- Grammatika M, Zimmerman W B, 2001, Microhydrodynamics offloatation process in the sea surface layer, Dynamics of Atmospheres and Ocean, 34, 327–348.
- Griffin D W, Gibson III C J, Lipp E K, Riley K, Paul III J H, Rose J B, 2003, Detection of Viral Pathogens by Reverse Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys, Applied and Environmental Microbiology, 65(9): 4118-4125.
- Gustafsson M E R, Franzén L G, 2000, Inland transport of marine aerosols in southern Sweden, Atmospheric Environment, 34, 313-325.
- Harvey G W, Burzell L A, 1972, A simple microlayer method for small samples. Limnology and Oceanogrphy, 11, 156–157.
- Klassen R D, Roberge PR, 1999, Aerosol transport modeling as an aid to understanding atmospheric corrosivity patterns, Materials & Design, 20 (4), 159–168.
- La Rosa G, Fratini M, Libera S, Iaconelli M, Muscillo M, 2012, Emerging and potentially emerging viruses in water environments, Ann Ist Super Sanità, Vol. 48 (4) 397-406.
- Liss P S, Duce R A, 1997, The Sea Surface and Global Change, Cambridge University Press, 519 pp.

- Mahy B W J, Van Regenmortel M H V, 2009, Desk Encyclopedia of General Virology, Elsevier.Oxford.
- Marks R, Kruczalak K, Jankowska K, Michalska M, 2001, Bacteria and fungi in air over the Gulf of Gdańsk and Baltic sea, Journal of Aerosol Science, 32, 237-250.
- Matthias-Maser S, Jaenicke R, 1995, The size distribution of primary biological aerosol particles with radii > 0.2 μm in an urban/rural influenced region, Atmospheric Research, 39 (4) 279–286.
- Moorthy K K, Satheesh S K, Murthy B V K, 1997, Investigations of marine aerosols over tropical Indian Ocean, Journal of Geophysical Research, 102, (18), 827 –842.
- Noble R T, Fuhrman J A, 1998, Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria, Aquatic Microbial Ecology, 14, 113–118.
- Pósfai M, Simonics R, Li J, Hobbs P V, Buseck P R, 2003, Individual aerosol particles from biomass burning in southern Africa: Compositions and size distributions of carbonaceous particles, Journal of GeophYsical Research, 108 (19).
- Prata C, Ribeiro A, Cunha A , Gomes N C M, Almeida A, 2011, Ultracentrifugation as a direct method to concentrate viruses in environmental waters: virus-like particles enumeration as a new approach to determine the efficiency of recovery, Journal of Microbiological Methods, 14 (1), 64-70.
- Roy C J, Milton D K, 2004, Airborne transmission of communicable infection - the elusive pathway, N. England Journal of Med, 350, 1710–1712.
- Silva J J, 1994, Circulação de água na Ria de Aveiro: Contribuição para o estudo da qualidade da água, PhD Thesis, Universidade de Aveiro.
- Suttle C A, Chen F, 1992, Mechanisms and Rates of Decay of Marine Viruses in Seawater, Applied and Environmental Microbiology, 58(11) 3721-3729.
- Tsai Y L, Sobsey M D, Sangermano L R, Palmer C J, 1993, Simple Method of Concentrating Enteroviruses and Hepatitis A Virus from Sewage and Ocean Water for Rapid Detection by Reverse Transcriptase-Polymerase Chain Reaction, Applied and Environmental Microbiology, 59 (10), 3488-3491.
- Vennema H, Bruin E, Koopmans M, 2002, Rational optimization of generic primers for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction, Journal of Clinical Virology, 25, 233-235.
- Verrault D, Moineau S, Duchaine C, 2008, Methods for Sampling of Airborne Viruses, Microbiology and Molecular Biology Reviews, 72 (3) 413-444.

- Villena C, El-Senousy W M, Abad F X, Pinto R M, Bosch A, 2003, Group A rotavirus in sewage samples from Barcelona and Cairo: emergence of unusual genotypes, Applied and Environmental Microbiology, 69 (7) 3919–3923.
- Woods J W, 2013, Waterborne Diseases of the Ocean, Enteric Viruses. Infectious Diseases, 381-398.
- Yu L, Wen Z, Li J, Yang W, Wang J, Li N, Lu J, 2010, Effects of different sampling solutions on the survival of bacteriophages in bubbling aeration. Aerobiologia, 26 (1) 75-82.

Chapter 5 - Influence of UV light exposure on viral abundance in aquatic system

Influence of UV light exposure on viral abundance in aquatic system

Catarina Prata, Francisco Coelho, Bruno Ladeiro, Ângela Cunha, Newton Gomes, Adelaide Almeida*

Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

*Corresponding author: E-mail: aalmeida@ua.pt, Tel.: +351234370350, Fax: +351234372587

ABSTRACT

Nowadays, one of the major issues related with global climate changes is the increase to ultraviolet light (UV) exposure of aquatic organisms. Over the next decades it is expected an increase in the exposure to damaging UV wave- lengths by all aquatic organisms with far-reaching ecological consequences. The aim of this work was to evaluate the influence of increased exposure to UV light in the abundance of two groups of RNA enteric viruses (rotavirus and enterovirus) present in the SML of marine systems.To reach this objective an experimental life support system (ELSS), composed by several sediment-water microcosms, was used. The ELSS mimics fundamental aspects of biological activity in marine ecosystems, namely photoperiod, light intensity and tidal cycles and allows the control of ultraviolet radiation (UVR), temperature, salinity and pH. After 57 days of incubation, surface microlayer (SML) samples were collected in microcosms exposed to UVR and in non-exposed microcosms. The abundance of two enteric viruses, a double-stand RNA (rotavirus) and a single-stand RNA (enteroviruses) was determined in UVR exposed and non-exposed samples. No significant reduction in rotavirus abundance ($p = 0.41 > 0.05$) was found in exposed samples, reduction of less than 1 log, but for enterovirus a significant reduction ($p = 0.03 < 0.05$) in abundance, around 2 log, was observed. The results indicate that RNA enteric viruses present different resistance to UVR and that this difference can be related with RNA type but other viral characteristics can be also involved in UVR resistance of these viruses. As in this study a damaging UV wave-length with fluence similar to that reaching the surface of the planet was used, an increase in the exposure to UV wave-lengths, associated to global climate changes, would influence the viral communities, namely those more exposed to UVR, such as SML viral communities of marine environments, with extensive ecological consequences.

Introdution

Climate change is increasingly recognized as a major risk to human health, with consequences occuring through direct and indirect routes and as a result of interactions with other changed environmental characteristics (Thomas et al, 2012). Interactions between climate change and stratospheric ozone will cause changes in the levels of ambient UVR in the future (Thomas et al, 2012). Ultraviolet light (UV) is electromagnetic radiation with wavelengths shorter than visible light that can induce damage in a variety of organisms.

UV light can be divided into different ranges including UVA (400 to 320 nm), which is weakly affected by stratospheric ozone, UVB (320 to 280 nm), which is strongly affected by ozone and UVC (280 to 100 nm) or short-range UV. UVC is considered to be germicidal, because at this range, UV light is mutagenic to microorganisms like bacteria and also for viruses (Kowalski et al, 2000; Hirneisen et al, 2010; Zepp et al, 2011).

UV light predominately damage the viral nucleic acid (Nuanualsuwan and Cliver 2003; Hirneisen et al, 2010), but at high enough doses (>1000 mWs/cm2) can also affect the capsid proteins, making the genome susceptible to DNases and RNases present in the environment (DeSena and Jarvis 1981; Smirnov and others 1983; Hirneisen et al, 2010). Enteric viruses that contain genomic RNA undergo successive morphological changes in the viral capsid when exposed to UV light (Katagiri et al 1967; Miller and Plagemann 1973; De Sena and Jarvis 1981; Hirneisen et al, 2010).

Many factors can enhance or decrease UV action, such as the type of nucleic acid of the viruses, viral proteins, type of host cell, viral strain, virus aggregation and experimental conditions (Fino and Kniel 2008; Hirneisen et al, 2010).

The effect of UV light on microorganisms depends on the UV dose applied, of its ability to protect itself from UV light and to repair damages (Sommer et al., 2001; Hu et al, 2011).

Because UV radiation from the sun is present in the environment, natural defense mechanisms, such as photoreactivation and dark repair, have evolved in bacteria and other microorganisms, allowing UV injured microorganisms to reverse UV-induced damages (Koivunen and Heinonen-Tanski, 2005; Quek and Hu, 2008; Hu et al, 2011). Furthermore, viruses with double-stranded DNA or RNA are more stable when exposed to UV, than single-stranded DNA or RNA viruses, because their undamaged DNA or RNA strand may serve as a template for repair by host enzymes (Fong and Lipp, 2005; Hirneisen et al, 2010). In fact, double stranded DNA (e.g. adenovirus) or RNA viruses (rotavirus) have been frequently responsible for recreational waterborne disease outbreaks (Gerba *et al*. 2002; Guo et al, 2010; Di Bartolo et al, 2011).

Other factors, such as water temperature, pH, association with sediments, predators, concentration of particular matter, salinity and raining (Le-Guyader *et al*. 1983, Chuan *et al*. 1983, Goyal *et al*. 1984, Yates *et al*. 1985, Griffin *et al*. 2003, Bosch *et al*. 2005; Bosch 2007; Fong *et al*. 2005, Bosch *et al*. 2006, Suttle 2007, Lugoli *et al*. 2009), also affect virus survival in the marine environment. These factors may act individually, or they may interact with each other, affecting viral survival in different ways (Cutler et al, 2012). Environmental parameters such as temperature, precipitation, pH, salinity, and UV light irradiation have been changed as consequence of global climate changes (Kim et al, 2010). These factors potentially have important consequences for aquatic microorganisms including waterborne human pathogens (Häder et al, 2011). It is expected that over the next decades, the exposure of aquatic microorganisms to damaging UV wave- lengths, particularly UV-B (280 to 320 nm) will increase with far-reaching ecological consequences (Andrady et al, 2010; Santos et al, 2012). The effects of UV-B (280–320 nm) on aquatic organisms depend on the dose of harmful radiation to which individuals are exposed, which in turn depends on the optical characteristics (i.e. UV-B transmittance) of the water body and organism positioning in the water column (Sommaruga 2003).

In this work, the influence of exposure to UV light in the abundance of twou groups of RNA enteric viruses (rotavirus and entetovirus) present in SML of an estuarine system was evaluated.

Material and methods

Microcosm general description

An ELSS, composed by several sediment-water microcosm (Figure 5.1), conceived by Coelho et al (2013) was used to evaluate UV light influence on viral concentration. The ELSS mimics fundamental aspects of biological activity in marine ecosystems, namely photoperiod, light intensity (including photosynthetically active radiation, PAR) and tidal cycles. Control of temperature, ultraviolet radiation (UVR), salinity and pH is also possible. The ELSS is divided into two frames of 16 microcosms (32 in total) (glass tanks 25 cm high, 28 cm length and 12.4 cm width, each with a maximum functional water volume of approximately 7 L) (Figure 5.1).

Sediment was collected at the east margin of Mira channel (40°37′ N, 8°44′ W), one of the main channels of the Ria de Aveiro lagoon (Portugal), in May 2011. The Ria de Aveiro is a shallow mesotidal coastal lagoon connected with the Atlantic Ocean through a single inlet, and characterized by four main channels with several secondary narrow channels, inner basins and extensive intertidal areas (Dias et al., 2001). Plexiglass cores of

undisturbed sediment samples (10 cm deep, 27 cm length and 10.6 cm width) were collected and each core transferred directly into individual microcosms of the ELSS. Microcosms containing the sediment were taken back to the laboratory and connected to the ELSS less than 2 h after sampling. The ELSS was operated continuously during 57 days (Coelho et al., 2013).

The ELSS system was programmed to simulate the specific characteristics of the estuarine system Ria de Aveiro, at the sampling site where and when sediment was collected. Salinity was adjusted to simulate the conditions recorded at the sampling location and kept constant (32.6 ± 1.5) during the experiment. The microcosms were exposed to a uniform semi-diurnal tidal regime, experiencing two high tides and two low tides daily. The minimum and maximum water levels above the sediment surface were approximately 5 cm (low tide) and 10 cm (high tide). During each tidal cycle about 50% of the water volume of each microcosm was exchanged (~1.5 l), using synthetic saltwater to compensate the water removed, thus simulating the water renewal percentage recorded for the sampling site. Water pH was adjusted to 8.0 and water temperature to 19 °C (Coelho et al., 2013). No water recirculation was employed in order to avoid cross contamination between experimental treatments and also laboratory artifacts that may be promoted by re-using the same water over time.

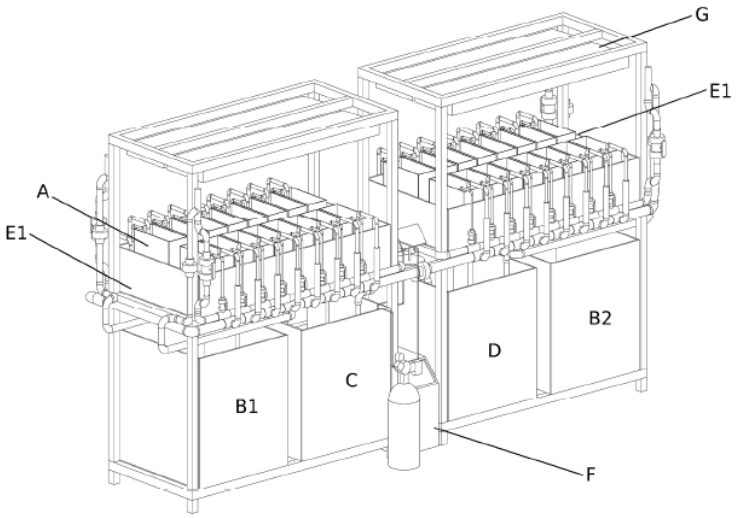

Figure 5.1: Representation of experimental life support system (ELSS) used in the UVR experiments (Coelho et al, 2013). A – independent microcosm; B – saltwater reservoir; C – acidified saltwater reservoir; D – normal pH saltwater reservoir, E – water bath; F – refrigerator; G – lightning system (a vinyl frame can be included to isolate the light from the luminaires), H - pH control system.

UV light control system

The ELSS is equipped with 4 Reef-SET®, (Rees, Germany) programmable luminaire system for diurnal light cycle and controlled UV simulation. Each luminaire holds four UV fluorescent tubes (SolarRaptor, T5/54W, Rees, Germany) and four full spectra fluorescent tubes (AquaLight, T5/54W/10000K, Bramsche, Germany) disposed alternately under a reflector.

The PAR value measured for the full spectrum fluorescent lamps is 260.50 ± 56.30 µmol photons m^{-2} s^{-1} and the UV-A (320–400 nm) and irradiance emitted by the UV lamps was 2875.91 ± 264.62 mW m^{-2}. To mimic summer photoperiod and light conditions at Portuguese latitudes during the experiment, a 14 h diurnal light cycle was simulated, with light intensity varying from 50 to 100% of the total fluorescent tube intensity. Since UV-A radiation is practically unaffected by changes in ozone depletion and plays an important role in biological systems, including photo-repair mechanisms (Bargagli, 2005), a similar amount of UV-A integrated irradiance was maintained constant among microcosms. The UV lamps were switched on for 4 hours a day at maximum intensity and UV-B component was filtered using a glass panel in the luminaire system. The UVR fluence used in the ELSS system corresponds to UV ligh that reach the surface of the planet in a summer day at the Portuguese latitudes. This UVB fluence is classified as a moderate intensity and is able of DNA damaging.

SML sampling

After the 57 days of incubation SML was collected using polycarbonate membranes, according to the method described by Cunliffe et al (2008). Briefly, a sterile polycarbonate membrane was placed directly onto the water surface, recovered with a tong and placed into a sterilized petri dish. SML samples were also collected in microcosms not exposed to UV light but exposed to PAR light during the 14 h diurnal light cycle. Three samples of each of three microcosms were collected and treated.

Nucleic acids extraction

Microorganisms were removed from polycarbonate membranes by using a mix of Zicornia and glass beads (0.10 g of 0.1 mm Zicornia beads, 0.20 g of 0.25-0.5 mm glass beads, 0.20 g of 0.75-1.0 mm glass beads and 2 glass beads of 2.85-3.45 mm). Polycarbonate membrane was cut and the beads were added in a 1.5 ml tube. One milliliter of lises buffer from the kit geneMAG-RNA/DNA kit, a magnetic RNA/DNA purification kit (Chemicell ™) was added to the tube which was centrifuged in Fastprep, at maximum velocity (6) for 40 s. Supernatant was removed to a clean tube and centrifuged for 1

minute. Nucleic acids were extracted from the supernatant using the geneMAG-RNA/DNA kit, a magnetic RNA/DNA purification kit (Chemicell ™), according to the instructions of the manufacturer. cDNA synthesis was made using a NZY First-Strand cDNA Synthesis Kit (Nzytech).

Enteric virus quantification

Quantification was made for rotavirus A and enterovirus, using a stepone plus 46 weels (Applied Biosystems) thermocycler and standards were obtained from a serial dilution of a suspension of each group tested, with a known initial number of copies.

Quantification of Rotavirus

Quantification of rotavirus was made by qPCR technique using the primers described by Villena *et al* (2003). Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0, 48µM of each primer (StabVida) (**VP6-3** 5': GCT TTA AAA CGA AGT CTT CAA C: 3' and **VP6-4** 5': GGT AAA TTA CCA ATT CTT CCA G: 3'). Rotavirus primers positions is found between the position 187 and position 166 of human strain Wa (accession number K02086).

The amplification was carried out for 40 cycles of 94ºC for 10 s, 50ºC for 30 s and 72ºC for 20 s after initial denaturation at 95ºC for 9 minutes. A final extension step was performed at 72ºC for 7 min.

Quantification of Enterovirus

Quantification of enterovirus was made by qPCR technique using the primers described by Beld *et al* (2004). Five microliters of sample were added to 20 µl of the reaction mixture which consisted of 2x iQ® SYBR® Green Super mix (2x reaction buffer with dNTPs, iTaq, DNA polymerase, 6mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) and 0, 48µM of each primer (StabVida) (**Entero 1** 5'- CCC TGA ATG CGG CTA AT -3' and **Entero 2** 5'- ATT GTC ACC ATA AGC AGC CA - 3'). The sequences of the primers used for amplification are located in the conserved 5' noncoding region of the EV sequence.

The amplification was carried out for 40 cycles of 95ºC for 10 s, 55ºC for 20 s and 72ºC for 30 s after initial denaturation at 95ºC for 10 minutes. A final extension step was performed at 72ºC for 5 min.

Statistical analysis

SPSS21 was used for statistical data analysis. The significance of reduction in viral abundance by UV light was assessed using one-way ANOVA. Data were tested for normal distribution (assessed by the Kolmogorov–Smirnov test) and homogeneity of variances (assessed by Levene's test). A value of $p < 0.05$ was considered significant.

Results

After exposure to UV light, the concentration of rotavirus in the exposed samples was slightly higher than in the control (Figure 5.2) but the difference, less than 1 log, corresponding to 33.87%, was not significant ($p = 0.41 < 0.05$). For Enterovirus the reduction after UV exposure was higher than that for rotavirus, around 2 log, corresponding to 79.43%, a value significantly different from that of the control ($p = 0.03 < 0.05$).

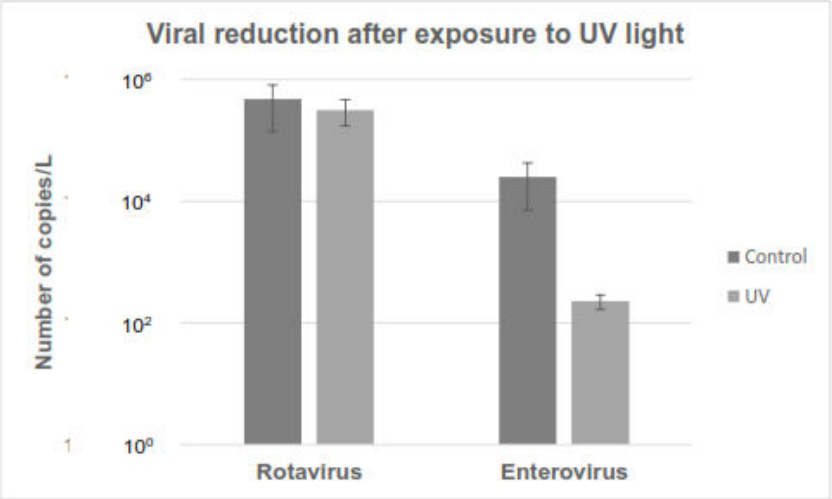

Figure 5.2: Reduction of rotavirus and enterovirus abundance after UV exposure. Control represents water sample without exposure to UV light. UV represents water samples exposed to UV light. Values represent the average of abundance values and error bars represent the standard deviation.

Discussion

The few studies testing the effect of UV light on viruses, evaluate viral infectivity and not changes in viral abundance. With this study, the effect of UVR on RNA enteric virus abundance was assessed, showing that aquatic RNA enteric viruses present different resistance to UVR. These differences may be related with nucleic acid type and other viral characteristics.

Viruses are the biological entity more abundant and are the reservoir of most of the genetic diversity in the marine environment (Comeau et al, 2007; Wegley et al, 2007), having a relevant role on the control of other microrganisms (e.g. bacteria), being a major force behind biogeochemical cycles, but also on the control of eukaryotic organisms such phytoplankton (Suttle, 2007) and mammalians (Suttle, 2007).

As in this study a damaging UV wavelength with fluence similar to that reaching the surface of the planet was used, an increase in the exposure to damaging UV wavelengths associated to global climate changes, would influence the viral communities, namely those more exposed to UVR, such as SML viral communities of marine environments, with far-reaching ecological consequences.

It is well known that UV light predominately damage the viral nucleic acid (Nuanualsuwan and Cliver 2003; Hirneisen et al, 2010) and that viruses with double-stranded DNA or RNA are more stable when exposed to UV than single-stranded DNA or RNA viruses. In fact, in this study, the double-stranded RNA rotavirus was more stable when exposed to UVR than the single-stranded RNA enterovirus. The ssRNA is more susceptible to RNases present in the environment than dsRNA (DeSena and Jarvis 1981; Smirnov and others 1983; Hirneisen et al, 2010).

On the other hand, it has been observed that enteric viruses that contain genomic RNA undergo successive morphological changes in the viral capsid when exposed to UV light (Katagiri et al 1967; Miller and Plagemann 1973; De Sena and Jarvis 1981; Hirneisen et al, 2010). The changed capsid proteins, make the genome susceptible to RNases present in the environment (DeSena and Jarvis 1981; Smirnov and others 1983; Hirneisen et al, 2010). As RNA viruses have less complex capsids (Costa et al., 2012) and the capsid proteins are affected by UVR, RNA genomes are easily exposed to UVR. Likewise, RNA viruses enclosing capsids with different complexity can also undergo different effects when under UVR. Rotavirus and enterovirus are non-lipid-coated icosahedral, but, contrarely to enterovirus, rotavirus are enclosed by a triple-layered capsid composed of two outer icosahedral layers surrounding an inner layer including different proteins (Bos et al, 2004; Grassi et al, 2009; Lawton et al, 2000).

Both, nucleic acid and capsid differences can explain the reduced number of outbreaks caused by enterovirus, when compared with rotavirus (Leveque *et al*. 2008). Rotavirus are responsible for a great number of outbreaks related with aquatic environment (Grassi *et al*. 2009; Di Bartolo et al, 2011).

Despite the reduction in viral abundance after UV exposition, namely for enterovirus, a great amount of viruses may resist to UV exposition and others may have their genomes affected but remain their infectivity. Another important issue related to viral resistance to UV light is the fact that viruses are genetically flexible, they may mutate quickly and, consequently, the emergence of UV resistant viruses as consequence of global climate changes can become a great problem.

Acknowledgements

The authors would like to thank the University of Aveiro and the Centre for Environmental and Marine Studies (CESAM) for funding the study. Financial support to C. Prata was provided by the Portuguese Foundation for Science and Technology (FCT) in the form of a PhD grant (SFRH/BD/33390/2008), São José Nascimento of University of Porto and Albert Bosh of University of Barcelona, for providing viral stocks used in this work as positive controls.

References

- Andrady A, Aucamp P J, Bais A F, Ballare C L, Björn L O, Bornman J F, Caldwell M, Cullen A P, Erickson D J, deGruijl F R, Häder D P, Ilyas M, Kulandaivelu G., Kumar H D, Longstreth J, McKenzie R L, Norval M, Paul N, Redhwi H H, Smith R C, Solomon K R, Sulzberger B, Takizawa Y, Tang X, Teramura A H, Torikai A, van der Leun J C, Wilson S R, Worrest R C, Zepp R G, 2010, Environmental effects of ozone depletion and its interactions with climate change: progress report, 2009, Photochemical and Photobiological Science, 9, 275–294.
- Bargagli R, 2005, Antarctic Ecosystems: Environmental Contamination, Climate Change, and Human Impact, Springer-Verlag, Berlin, Germany.
- Beld M, Minnaar R, Weel J, Sol C, Damen M, vanBreda A, Boom R, 2004, Highly Sensitive Assay for Detection of Enterovirus in Clinical Specimens by Reverse Transcription-PCR with an Armored RNA Internal Control, Journal of Clinical Microbiology, 42 (7), 3059–3064
- Bos T J, Somers K D, 2004, USMLE Road Map: Microbiology & Infectious Disease. McGraw-Hill Professional.
- Bosch A, Abad F X, Pintó R M, 2005, Human pathogenic viruses in the marine environment, Oceans and Health: Pathogens in the Marine Environment, 109-131
- Bosch A, Abad F X, Pintó R M, 2006, Survival and Transport of Enteric Viruses in the Environment, Viruses in Foods Food Microbiology and Food Safety, 151-187.
- Bosch A, 2007, Viruses Ocurrence and Survival in the Environmental waters, In “Human Viruses in water”, First Edition, Elsevier pp 91-108.
- Chuan S, Ellender R D, Johnson R A, Howell F G, 1983, Elution of Viruses from Coastal Sediments, Applied and Environmental Microbiology, 46(4), 797-804.
- Coelho F J R C, Rocha R J M, Pires A C C, Ladeiro B, Castanheira J M, Costa R, Almeida A, Cunha Â, Lillebø A I, Ribeiro R, Pereira R, Lopes I, Marques C, Moreira-Santos M, Calado R, Cleary D F R, Gomes N C M, 2013, Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities, Global Change Biology, doi:10.1111/gcb.12227
- Comeau A M, Hatfull G F, Krisch H M, Lindell D, Mann N H Prangishvili D, 2008, Exploring the prokaryotic virosphere, Research in Microbiology, 159, 306 – 313.
- Costa L A S, 2012, Photoinactivation of viruses by porphyrins, PhD thesis, Universidade de Aveiro.

- Cunliffe M, Schäfer H, Harrison E, Cleave S, Upstill-Goddard R, Murrell J C, 2008, Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary, ISME Journal, 2, 776–789
- Cutler T D, Wanga C, Hoff S J, Zimmerman J J, 2012, Effect of temperature and relative humidity on ultraviolet (UV254) inactivation of airborne porcine respiratory and reproductive syndrome virus, Veterinary Microbiology, 159, 47–52
- De Sena J, Jarvis D, 1981, Modification of the poliovirus capsid by ultraviolet light. Can Journal of Microbiology, 27, 1185–93.
- Dias J M, Lopes J F, Dekeyser I, 2000, Tidal propagation in Ria de Aveiro lagoon, Portugal. Physical and Chemical Earth; 25, 369-74
- Di Bartolo I, Monini M, Losio M N, Pavoni E, Lavazza A, Ruggeri F M, 2011, Molecular Characterization of Noroviruses and Rotaviruses Involved in a Large Outbreak of Gastroenteritis in Northern Italy, Applied and Environmental Microbiology, 77(15) 5545-5548.
- Fino V R, Kniel K E, 2008, UV light inactivation of hepatitis A virus, aichi virus and feline calicivirus on strawberries, green onions, and lettuce, Journal of Food Pro, 71, 908–13.
- Fong T-T, Lipp E K, 2005, Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools, Microbiology and Molecular Biology reviews, 69 (2) 357-371.
- Gerba C P, Gramos D M, Nwachuku N, 2002, Comparative Inactivation of Enteroviruses and Adenovirus 2 by UV Light, Applied and Environmental Microbiology, 68 (10): 5167–5169.
- Goyal S M, Adams W N, O'Malley M L, Lear D W, 1984, Human Pathogenic Viruses at Sewage Sludge Disposal Sites in the Middle Atlantic Region, Applied and Environmental Microbiology, 48 (4), 758-763.
- Grassi T, Bagordo F, Ídolo A, Lugoli F, 2009, Rotavirus detection in environmental water samples by tangential flow ultrafiltration and RT-nested PCR, Environmental Monitoring Assess.
- Griffin D W, Gibson III C J, Lipp E K, Riley K, Paul III J H, Rose J B, 2003, Detection of Viral Pathogens by Reverse Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys, Applied and Environmental Microbiology, 65(9) 4118-4125.

- Guo H, Chu X, Hu J, 2010, Effect of Host Cells on Low- and Medium-Pressure UV Inactivation of Adenoviruses, Applied and Environmental Microbiology, 76 (21) 7068–7075.
- Häder D-P, Helbling E W, Williamson C E, Worrest R C, 2011, Effects of UV radiation on aquatic ecosystems and interactions with climate change, Photochemical and Photobiological Science, 10, 242-260
- Hirneisen K A, Black E P, Cascarino J L, Fino V R, Hoover D G, Kniel K E, 2010, Viral Inactivation in Foods: A Review of Traditional and Novel Food-Processing Technologies, Comprehensive reviews in food science and food safety, 9, 3-20.
- Hu X, Geng S, Wang X, Hu C, 2011, Inactivation and Photorepair of Enteric Pathogenic Microorganisms with Ultraviolet Irradiation, Environmental Engineering Science, 29 (6) 549-553.
- Katagiri S, Hinuma Y, Ishida N, 1967, Biophysical properties of poliovirus particles irradiated with ultraviolet light, Virology, 32, 337–43.
- Kim J, Park J, Kim P-G, Lee C, Kyungho K C, 2010, Implication of global environmental changes on chemical toxicityeffect of water temperature, pH, and ultraviolet B irradiation on acute toxicity of several pharmaceuticals in Daphnia magna, Choi. Ecotoxicology, 19:662–669
- Koivunen J, Heinonen-Tanski H, 2005, Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments, Water Research, 39, 1519.
- Kowalski W J, Bahnfleth W P, Witham D L, Severin B F, Whittam T S, 2000, Mathematical modeling of ultraviolet germicidal irradiation for air disinfection, Quant Microbiology, 2, 249–70.
- Lawton J A, Estes M K, Prasad B V V, 2000, Mechanism of genome transcription in segmented dsRNA viruses, Advances in Virus Research, 55: 185–214, IN1–IN8, 215–229.
- Le-Guyader F L, Apaire-Marchais V, Brillet J, Billaudel S, 1983, Use of Genomic Probes To Detect Hepatitis A Virus and Enterovirus RNAs in Wild Shellfish and Relationship of Viral Contamination to Bacterial Contamination, Applied and Environmental Microbiology, 59(11) 3963-3968.
- Leveque N, Laurent A, 2008, A Novel Mode of Transmission for Human Enterovirus Infection Is Swimming in Contaminated Seawater: Implications in Public Health and in Epidemiological Surveillance, Clinical Infection, 47, 624–626.

- Lugoli F, Leopizzi M I, De Donno A, 2009, Viral Indicators of Hygienic-Sanitary Quality: Detection of Somatic Coliphages in the Southern Adriatic Sea, Water Qual Expo Health, 1, 171-177.
- Miller R, Plagemann P, 1973, Effect of ultraviolet light on mengovirus: formation of uracil dimers, instability and degradation of capsid, and covalent linkage of protein to viral RNA, Journal of Virology, 13, 729–39.
- Nuanualsuwan S, Cliver D O, 2003, Capsid Functions of Inactivated Human Picornaviruses and Feline Calicivirus, Applied and Environmental Microbiology, 69 (1) 350-357.
- Quek P H, Hu J Y, 2008, Indicators for photoreactivation and dark repair studies for following ultraviolet disinfection, Journal of Industrial Microbiology Biotechnology, 35, 533.
- Santos A L, Oliveira V, Baptista I, Henriques I, Gomes N C M, Almeida A, Correia A, Cunha A, 2012, Effects of UV-B Radiation on the Structural and Physiological Diversity of Bacterioneuston and Bacterioplankton, Applied and Environmental Microbiology, 78 (6) 2066
- Smirnov Y A, Rodrigues-Motto MP, Famadas MT, 1983, Protein-RNA interaction in encephalomyocarditis virus as revealed by UV light-induced covalent linkages, Journal of Virology, 45, 1048–55.
- Sommaruga R, 2003, UVR and its effects on species interactions, Organisms and Ecosystems, Edited by E.W. Helbling and H. Zagarese, pp. 485-508, The Royal Society of Chemistry, Cambridge, UK.
- Sommer R, Pribil W, Appelt S, Gehringer P, Eschweiler H, Leth H, Cabaj A, Haider T, 2001, Inactivation of bacteriophages in water by means of non-ionizing (UV253.7nm) and ionizing (gamma) radiation: A comparative approach, Water Research, 35, 3109.
- Suttle C A, 2007, Marine viruses — major players in the global ecosystem, Nature reviews – microbiology, 5: 801-811.
- Thomas P, Swaminathan A, Lucas R M, 2012, Climate change and health with an emphasis on interactions with ultraviolet radiation: a review, Global Change Biology, 18 (8) 2392–2405.
- Villena C, El-Senousy W M, Abad F X, Pinto R M, Bosch A, 2003, Group A Rotavirus in Sewage Samples from Barcelona and Cairo: Emergence of Unusual Genotypes, Applied and Environmental Microbiology, 69 (7) 3919-3923.

- Wegley L, Edwards R, Rodriguez-Brito B, Liu H, Rohwer F, 2007, Metagenomic analysis of the microbial community associated with the coral Porites astreoides, Environmental Microbiology, 9 (11) 2707–2719.
- Yates M V, Gerba C P, Kelley L M, 1985, Virus persistence in groundwater, Applied and Environmental Microbiology, 49(4): 778-781.
- Zepp R G, Erickson III D J, Paulc N D, Sulzbergerd B, 2011, Effects of solar UV radiation and climate change on biogeochemical cycling: interactions and feedbacks, Photochemical and Photobiology Science, 10, 261-279.

Chapter 6 – Final Considerations

Although there is a great amount of viruses in the aquatic system, only a few number is important for human health. As pathogenic viruses are present in low numbers in the aquatic environment, samples must be concentrated to allow efficient detection of very few viruses in a large volume of water (Schwab et al, 1995). However, there is no perfect viral concentration method for water samples (Bosch et al, 2008; Albinana-Gimenez et al, 2009), and it should be chosen to achieve the aim of the work.
In this work, it was compared the ultracentrifugation and the organic flocculation methods and it was detected that ultracentrifugation method can recover about 50% more viruses in recreational waters than the organic flocculation method, one of the most currently used approache to concentrate viruses from environmental waters (Calgua et al, 2008; Guttman-Bass and Armon, 1983; Guttman-Bass and Nassen, 1984; Shields and Farrah, 1986; virobathe, 2011).

Besides the higher recovery of viruses obtained by the ultracentrifugation method, compared with the organic flocculation method, the first presents some other advantages that made it the best method to use in this work: is a simple and fast method, does not interfere with viral community structure, no chemicals are added to the sample avoiding PCR inhibition and it can reduce the final volume of sample to few microliters.

In virology there is still scarce information in some areas. Nowadays, much is already known about the viruses present in the aquatic system that represent a health hazard for humans, but little is known about their natural reservoirs, namely the surface microlayer (SML), and their transmission to the atmosphere through aerosols, mainly because of the difficulty in collecting and analyzing airborne virus (Verrault et al, 2008).
The sea SML corresponds to the interface between the surface of the water and the air and is known to accumulate particles and microorganisms and plays a crucial role in exchange processes of gases and matter across the air-water interface. The gases and matter transferred from the SML are transported across the air-water interface by aerosols. So, aerosols composition depends on SML composition.
With this work it was found that SML acts as a major source of microorganisms to the atmosphere and aerosols constitute an important mechanism of viruses dispersion.
In order to form and collect aerosols in field work, a protocol was developed. Polycarbonate membranes (0.45 μm) were tested to filtrate the air used to form aerosols, miliQ water and 1xPBS were compared as a collection medium and two times (30 minutes and 1 hour) of aerosols formation were tested. The polycarbonate

membranes were efficient in removing viruses from the air, with a reduction of about 80% and they do not interfere with viral community, since values of VLP between 10^9 and 10^{10} VLP L^{-1} (data not shown), which is according to the literature (viral concentration range between 10^7 L^{-1} and 10^{11} L^{-1} in coastal areas (Wommack and Colwell, 2000) were detected. The collection medium chosen was miliQ water, mainly because of the background fluorescence of 1x PBS in the epifluorescence microscopy. The time needed to form aerosols in laboratory was of 1 hour. So, the protocol established for aerosols formation and recovery in field work was formation of aerosols for 1 hour, with air filtered by a polycarbonate membrane bubbling in the water sample and collection of the aerosols formed in miliQ water.

The five groups of enteric viruses tested were detected in water and aerosols in both the marine and the brackish water zones. Viral abundance obtained was according to which is indicated in the literature and the patterns of variation were also according to that described in the literature. Rotavirus and Enterovirus presented a peak in cold months, as in other studies (2000; Guix et al, 2002; Bosch et al, 2008), and HAV presented similar density in all sampling dates. Rotavirus and Enterovirus ranged between 10^2 – 10^7 copies L^{-1} and HAV range between 10^1 – 10^2 copies L^{-1}. The values obtained in other works described in the literature were of 10^1 to 10^8 copies L^{-1} for Rotavirus (Li et al, 2010; Chigor and Okoh, 2012; Vieira et al, 2012), 10^1 to ^{7} copies L^{-1} for Enterovirus (Zhang et al, 2010; Chigor and Okoh, 2012; Aslan et al, 2013) and 10^1 to 10^9 copies L^{-1} for HAV (Chigor and Okoh, 2012; Keuckelaere et al, 2013).

The variation found in viral abundance is better explained by temperature and nitrites plus nitrates concentration, decreasing viral concentration with the increase in temperature and incrasing with the increase in nitrites plus nitrates concentration. This suggests that enteric viruses have mainly terrestrial origin reaching the marine environment through runoff namely during the winter months.

Enteric viruses were more abundant in the SML and less abundant in the aerosols, with significant differences between the water layers and the aerosols ($p < 0.05$), both in the marine and in the brackish water zones.Enteric viruses were about 6 and 18 times more abundant in the SML than in UW, in marine and brackish water zones, respectively and 14.63 and about 46 times more abundant in the SML than in the aerosols, for marine and brackish water zones, respectively. These results are according the literature since virus concentration in the air is low (Després et al, 2011) and the SML is generally enriched in microrganisms (Cunliffe et al, 2011) and is agreement to the work of Aller et al (2005), that indicates that the SML acts as a major

source of microorganisms to the atmosphere and aerosols constitute an important mechanism of viral dispersal to long distances.

Viral survival may vary, depending on viral type. However, there are several factors that may affect that survival, like exposure to UV light, water temperature, pH, salinity, adsorption to sediments, predators, presence of particular matter, salinity and raining (Le-Guyader *et al*. 1983, Chuan *et al*. 1983, Goyal *et al*. 1984, Yates *et al*. 1985, Griffin *et al*. 2003, Bosch *et al*. 2005; Bosch 2007; Fong *et al*. 2005, Bosch *et al*. 2006, Suttle 2007, Lugoli *et al*. 2009). In this work the influence of exposure to UV light on viral abundance was tested, but usually the studies about the effect of UV exposition on viruses test only viral infectivity and not changes in viral abundance. The results of this study showed that for Rotaviruses no significant reduction was found in viral abundance but a significant reduction was found for Enterovirus ($p = 0.03 < 0.05$). The difference observed between the two viruses can be related to RNA type, dsRNA in

Rotavirus and ssRNA in Enterovirus, but also with the structure of the viral capsid, a three-layered capsid in Rotavirus and a single capside in Enterovirus. It is well known that dsDNA are more resistant to environmental factor such as UVR, and as a complex capsid difficult the RNA damage mechanism, Rotavirus is more resistant to UVR than Enteroviruses. This may explain the reduced number of Enterovirus waterborne outbreaks relatively to Rotavirus (Leveque *et al*. 2008).

The development of scientific work for a PhD thesis must generate knowledge that contributes in some way to create value, whether social, economic or environmental.
Since enteric viruses have low infectious dose, being responsible for numerous hospitalizations, due to gastroenteritis, it is extremely important to know how these viruses can be transmitted to humans, in order to minimize this scourge. The finds that SML is a reservoir of enteric viruses in the marine environment and that there is potential for their transference from water to air. This is an important knowledge in preventing waterborn viral infection. This work provides the basis for future studies. Future work will pass to:

- Determine the percentage of the UV light exposed remaining viruses that are still capable of successfully infect host cells.
- The time and UV necessary to reduce significantly the abundance of all enteric viruses tested.

- Determine the distance that enteric viruses travel inside aerosols maintaining their viability.
- Determine the conditions in the aerosols transport that interfere with viral infectivity (time inside the aerosol, influence of external factors, like UV light, humidity, etc.)

References

- Albinana-Gimenez N, Miagostovich M P, Calgua B, Huguet J M, Matia L, Girones R, 2009, Analysis of Adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants, Water Research, 43, 2011–2019.
- Aller J Y, Kuznetsova M R, Jahns C J, Kemp P F, 2005, The sea surface microlayer as a source of viral and bacterial enrichment in marine aerosols, Aerosol Science, 36, 801–812
- Aslan A, Xagoraraki I, Simmons F J, Rose J B, Dorevitch S, 2011, Occurrence of adenovirus and other enteric viruses in limited-contact freshwater recreational areas and bathing waters, Journal of Applied Microbiology, 111, 1250–1261.
- Bosch A, Abad F X, Pintó R M, 2005, Human Pathogenic Viruses in the Marine Environment, Oceans and Health: Pathogens in the Marine Environment, 109-131
- Bosch A, Abad F X, Pintó R M, 2006, Survival and transport of enteric viruses in the environment, In “Viruses in Food”, S. M. Goyal (Ed.), Food Microbiology and Food Safety Series, Springer, New York, pp 151-187.
- Bosch A, 2007, Viruses Ocurrence and Survival in the Environmental waters, In “Human Viruses in water”, First Edition, Elsevier, pp 91-108.
- Bosch A, Guix S, Sano D, Pinto R M, 2008, New tools for the study and direct surveillance of viral pathogens in water, Current Opinion in Biotechnology, 19, 295–301.
- Calgua B, Mengewein A, Grunert A, Bofill-Mas S, Clemente- Casares P, Hundesa A, Wyn-Jones A P, Lopez-Pila J M, Girones R, 2008, Development and application of a one-step low cost procedure to concentrate viruses from seawater samples, Journal of Virological Methods, 153, 79–83.
- Chigor V N, Okoh A I, 2012, Quantitative RT-PCR Detection of Hepatitis A Virus, Rotaviruses and Enteroviruses in the Buffalo River and Source Water Dams in the Eastern Cape Province of South Africa, International Journal of Environmental Research in Public Health, 9, 4017-4032.

- Chuan S, Ellender R D, Johnson R A, Howell F G, 1983, Elution of Viruses from Coastal Sediments, Applied and Environmental Microbiology, 46(4): 797-804.
- Cunliffe M, Upstill-Goddard R C, Murrell J C, 2011, Microbiology of aquatic surface microlayers, FEMS Microbiological Reviews, 1–14.
- Després V R, Huffman J A, Burrows S M, Hoose C, Safatov A S, Buyak G, Fröhlich-Nowoisky J, Elbert W, Andreae M, Pöschil U, Jaenicke R, Tellus B, 2012, Primary biological aerosol particles in the atmosphere: a review, Chemical and Physical Meteorology, 64, 15598.
- Fong T-T, Lipp E K, 2005, Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools, Microbiology and Molecular Biology reviews, 69 (2) 357-371.
- Goyal S M, Adams W N, O'Malley M L, Lear D W, 1984, Human Pathogenic Viruses at Sewage Sludge Disposal Sites in the Middle Atlantic Region, Applied and Environmental Microbiology, 48 (4), 758-763.
- Griffin D W, Gibson III C J, Lipp E K, Riley K, Paul III J H, Rose J B, 2003, Detection of Viral Pathogens by Reverse Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys, Applied and Environmental Microbiology, 65(9): 4118-4125.
- Guttman-Bass N, Armon R, 1983, Concentration of Simian Rotavirus SA-11 from tap water by membrane filtration and organic flocculation, Applied and Environmental Microbiology, 45(3), 850– 855.
- Guttman-Bass N, Nassen A, 1984, Simultaneous concentration of four enterovirus from tap, waste and natural waters, Applied and Environmental Microbiology, 47 (6), 1311–1315.
- Le-Guyader F L, Apaire-Marchais V, Brillet J, Billaudel S, 1983, Use of Genomic Probes To Detect Hepatitis A Virus and Enterovirus RNAs in Wild Shellfish and Relationship of Viral Contamination to Bacterial Contamination, Applied and Environmental Microbiology, 59(11) 3963-3968.
- Leveque N, Laurent A, 2008, A Novel Mode of Transmission for Human Enterovirus Infection Is Swimming in Contaminated Seawater: Implications in Public Health and in Epidemiological Surveillance, Clinical Infection, 47, 624–626.
- Lugoli F, Leopizzi M I, De Donno A, 2009, Viral Indicators of Hygienic-Sanitary Quality: Detection of Somatic Coliphages in the Southern Adriatic Sea, Water Qual Expo Health, 1, 171-177.

- Schwab K J, De Leon R, Sobsey M, 1995, Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A viruses, and norwalk viruses by reverse transcription-PCR, Applied and Environmental Microbiology, 61 (2) 527–531.
- Shields P A, Farrah S R, 1986, Concentration of viruses in beef extract by flocculation with ammonium sulfate, Applied and Environmental Microbiology, 51(1) 211–213.
- Suttle C A, 2007, Marine viruses — major players in the global ecosystem, Nature, 5, 801-812.
- Verrault D, Moineau S, Duchaine C, 2008, Methods for Sampling of Airborne Viruses, Microbiology and Molecular Biology Reviews, 72 (3) 413-444.
- Vieira C B, Mendes A C O,Guimarães F R, Fumian T M, Leite J P G, Gaspar A M C, Miagostovich M P, 2012, Detection of enteric viruses in recreational waters of an urban lagoon in the city of Rio de Janeiro, Brazil, Mem Inst Oswaldo Cruz, 107(6) 778-784.
- Virobathe, www.virobathe.org, 2011.
- Wommack K E, Colwell R R, 2000, Virioplankton: virus in aquatic ecosystems, Microbiol. Mol. Biol. Rev., 64(1) 69–114.
- Yates M V, Gerba C P, Kelley L M, 1985, Virus persistence in groundwater, Applied and Environmental Microbiology, 49(4): 778-781.
- Zhang M, Zhao H, Yang J, Jiang S, Cai B, 2010, Detection and quantification of enteroviruses in coastal seawaters from Bohai Bay, Tianjin, China, Journal of Environmental Sciences, 22 (1) 150–154.